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**The Effects of Luminal
Nitric Oxide on
Gastro-Oesophageal Motility**

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**Thesis submitted for the Degree of
Doctor of Medicine**

**University of Glasgow
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December 2006

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Abstract

- Over the last few decades, the epidemiology of diseases of the proximal gastrointestinal tract has been changing. The incidence of gastric adenocarcinoma in the United Kingdom has decreased, with squamous carcinoma of the oesophagus remaining relatively static. Adenocarcinoma of both the oesophagus and gastro-oesophageal junction (including the cardia) have increased greatly.
- The risk of oesophageal adenocarcinoma has been linked with gastro-oesophageal reflux disease. Short-segment reflux at the cardia has also been postulated as a risk factor for cancers at this site.
- The factors that predispose a particular person to more severe reflux or put them at an increased risk of mucosal damage are poorly understood. Inherent physiological and anatomical parameters will play their roles, with the role of environmental factors often being overlooked.
- The increase in dietary nitrate consumption, following the post-war intensive farming practices with nitrogenous fertilizers, has been proposed as a risk factor for the increase in several human cancers.
- The entero-salivary recirculation of dietary nitrate has been examined by several groups. It has demonstrated the generation of high quantities of nitrite in swallowed human saliva. The subsequent luminal chemistry will cause nitrous acid (NA) to be formed in a healthy acid secreting stomach. In the additional presence of ascorbic acid, instantaneous formation of luminal nitric oxide (NO) at the gastric

cardia takes place. Nitrous acid is also capable of forming long acting NO-donor compounds, by reacting with ingested dietary thiols.

- Nitric oxide is a ubiquitous smooth muscle relaxant. It is involved in oesophageal peristalsis, lower oesophageal sphincter (LOS) and gastric relaxation and also the co-ordination of transient lower oesophageal sphincter relaxations (TLOSRs).
- A series of pilot studies, in healthy volunteers, were designed to investigate the physiological effects on gastro-oesophageal motility.
- A standard water perfused manometry system was adapted, and validated, to deliver a test solution containing NO, whilst also recording pressure data from the proximal gastro-intestinal tract.
- The profile of distribution of the delivered NO solutions was characterised by a series of silastic tube experiments. A segmented silastic tube was taped to the manometry catheters used for each experiment. The NO was delivered as it would be in each experiment.
- The segments trapped NO as nitrite, which were then analysed. They showed a pattern of NO distribution similar to the fasting data seen with a luminal NO probe. The post-prandial model showed evidence of NO reflux into the oesophagus and also NO concentrations comparable to those following ingestion of oral nitrate.
- Smaller amounts of nitrite are present in saliva in the fasting state. In our model of the subsequent formation of NO at the gastric cardia, there was no effect on the resting pressure of the LOS. There was also no effect on the length of the LOS.

- At high concentrations (>4mM), NO can come out of solution to form gaseous NO. *In vitro* experiments demonstrated that at the experimental concentrations used, and also at 37°C, no significant quantities of NO gas were formed, excluding the possibility of intra-gastric gaseous distension as a confounding factor in our studies.
- Post-prandial studies were performed in 15 healthy volunteers. Initially, the manometric effects of a meal on the LOS were observed. Subsequently, double-blinded solutions were perfused into the lumen at the gastro-oesophageal junction. Hydrochloric acid, NA and NO solutions, all at the upper physiological limit, were perfused and recordings made.
- The effect of a meal on the LOS, was to cause a reduction in the total sphincter length with a preferential loss of the abdominal portion. There also appeared to be a slight elevation of the diaphragm, but no shortening of the oesophagus.
- Nitric oxide caused an increase in TLOSRS and reflux at the distal oesophagus. Nitrous acid caused a reduction in intra-gastric pressure during the infusion. It caused no alteration to the acid reflux profile.
- No solutions caused any major alterations to oesophageal peristaltic parameters following 'dry' swallows.
- These findings support the need for further research into this area. The threshold for effects caused by nitrite chemistry must be addressed. Also, these studies were performed in healthy volunteers. It would be reasonable to examine the effects of this luminal chemistry on those with pre-existing upper gastrointestinal diseases such as

hiatus hernias, reflux oesophagitis and Barrett's oesophagus. Repeat studies looking more simply at the effects of swallowed nitrite on oesophageal acid exposure would be enlightening.

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Abbreviations:

AL – abdominal sphincter length

CCK – cholecystokinin

cGMP – cyclic-guanilyl mono-phosphate

GABA – gamma-amino butyric acid

GOJ – gastro-oesophageal junction

GORD – gastro-oesophageal reflux disease

GSNO – S-nitrosoglutathione

GTP – guanylyl tri-phosphate

HPZ – high pressure zone

iNOS – inducible nitric oxide synthase

IGP – intragastric pressure

LNMA – L-NG-monomethyl arginine

LOS – lower oesophageal sphincter

MEEP – maximum end expiratory pressure

NA – nitrous acid

NERD – non-erosive reflux disease

NO – nitric oxide

RIP – respiratory inversion point

SCJ – squamo-columnar junction

SMPT – slow motorised pull-through

SNAP – S-nitroso-N-acetylpenicillamine

TLOSR – transient lower oesophageal sphincter relaxation

Acknowledgements

In completing this work I would like to extend my gratitude to many people involved in these studies.

Professor McColl, my supervisor, has given excellent guidance for these studies and advice on writing, both during the research period and afterwards as well.

Angela Wirz, Clinical Research Sister, helped with both the clinical and laboratory studies.

Drs Simon Dover and Richard Park, supervising Clinical Consultants, who gave me great encouragement to write up this thesis.

Dr Andrew Manning, my brother, who helped extensively as a healthy volunteer during the testing of all these systems, and as part of the actual studies.

Seonaid Manning, my wife, for her support and also taking part as a healthy volunteer. And finally, to my other family members, for being there and supporting both my research and career over the past years.

**The potential effects of the acidification
of salivary nitrite on the physiology
of the upper gastrointestinal tract**

Chapter 1

Thesis aims

This thesis brings together a set of experiments to examine the potential physiological effects of luminal nitric oxide, generated from salivary nitrite, on the oesophagus and lower oesophageal sphincter of humans. A review of the literature regarding gastro-oesophageal reflux disease and nitrite chemistry is undertaken. Subsequent chapters describe the design and testing of the experimental equipment both *in vitro* and *in vivo*, prior to undertaking the two studies. The first study analyses the effects of these chemicals, in healthy subjects, in the fasted state and then this is followed by a study examining the effects following a meal.

1.1 Introduction:

The human oesophagus and stomach are common sites of symptoms and disease. In particular, the incidence of oesophageal adenocarcinoma has increased by about 600% since 1975 exceeding the relative increase over that time period of melanoma (1). In Iran, which has a high prevalence of *H. pylori* infection, the pattern of upper gastrointestinal malignancy has changed recently from one of predominantly antral adenocarcinoma in the 1970's (2) to the gastric cardia in 2000 (3).

Gastro-oesophageal reflux disease (GORD) is extremely prevalent in the adult population of the Western world. Approximately 15% of adults will experience symptoms on a weekly basis, and 5% daily, although only a small proportion of these will consult their General Practitioner (4). A greater frequency of reflux symptoms is associated with an increase in the relative risk of developing oesophageal adenocarcinoma (5). Although, in contradiction, oesophageal inflammation and oesophagitis do not always correlate with heartburn and reflux symptoms (6).

1.2 Anatomy:

An understanding of the anatomy is important in appreciating the pathophysiology that occurs. Grossly these organs comprise two hollow tubes separated by a muscular valve or sphincter. This separation is important in keeping the two specialised environments apart. The primary function of the oesophagus is the propulsion of foodstuffs to the stomach, which is achieved by co-ordinated peristalsis of the musculature. The oesophagus passes through the thorax in the posterior mediastinum and has

both an outer longitudinal and inner circular layer of muscle. In its upper quarter the fibres are those of striated muscle, with an increasing mixture of smooth muscle in the next quarter. The lower half is comprised entirely of smooth muscle.

Careful dissection of the muscle layers has shown that the fibres are organised in a helical fashion. Those in the circular layer form a tight helix and those in the longitudinal layer an elongated one (7). There are several important layers lining this muscular tube. The mucosa comes into direct contact with the luminal environment. The mucosa comprises an epithelium, lamina propria and muscularis mucosa. In the human oesophagus this is a partially keratinised stratified squamous epithelium measuring 300micrometres thick. The lamina propria is deep to this and is primarily a connect tissue and vascular layer. The muscularis mucosae is a 200-400micrometres thick layer of smooth muscle, with its proximal insertion at the cricoid cartilage (7). The muscle bundles are orientated longitudinally in the oesophagus and become significantly thicker at the gastro-oesophageal junction (GOJ) (8). Here, the muscularis is thicker than that of the stomach, and can be mistaken for the muscularis propria on biopsy. The orientation changes on passage to the stomach showing both a circular and longitudinal arrangement. The elastic submucosa with glands completes the picture, lying between the two muscle layers (9);(10) – see Figure 1.1.

A broader band of circular muscle exists at the lower end of the oesophagus and is often termed the lower oesophageal sphincter (LOS). Definitions of this area are very complicated as no definite anatomical sphincter exists. It's location can be described by measurements of the pressure it exerts, but in different states of disease, such as hiatus hernias,

these figures are unreliable. This is true for mucosal definitions of the sphincter, which are also subject to variations in disease (11). Anatomical descriptions such as the peritoneal reflection from the stomach to the diaphragm are only of use in surgical resection specimens (12).

In a fasting state the oesophagus is a collapsed structure (13), devoid of air or fluid, with the mucosa thrown in to longitudinal folds. Endoscopic insufflation of air distorts this, giving a false impression of the resting state. Normally the inner oesophageal surface has a pink-white colour. The upper margin of the diaphragmatic indentation can be seen at endoscopy and is one landmark used as the GOJ. Another is the proximal folds of the gastric mucosa, which is said to approximate to the muscular junction (14). The mucosa of a healthy oesophagus differs to that of the stomach. In contrast to a healthy oesophagus, the stomach is lined by a columnar epithelium. The change from one epithelium to another is quite distinct and appears as a serrated border or 'z-line'.

Small projections of gastric epithelium up to 5mm long can be seen rising up into the squamous epithelium, and are often asymmetrical. This mucosal definition of the GOJ differs to the muscular definition, as the squamo-columnar junction (SCJ) often lies within the high pressure zone of the LOS. That is to say, in the presence of a normal size cardia, almost the whole of the lower half of the mucosa within the LOS is of an acid secreting type (15). These descriptions show the differing opinions and conflicts that can exist between anatomists, physiologists, pathologists, surgeons and gastroenterologists, when describing an important area of disease; the location and features of which are paramount in disease classification and management.

1.3 Swallowing and reflux:

Incompetence of the LOS is one mechanism that allows the harmful gastric juices to cause symptoms and damage to the lower oesophageal mucosa. Although this is a well-understood concept, the actual causes of sphincter failure are poorly understood. Acid reflux occurs as a consequence of the retrograde movement of the luminal contents of the stomach back into the oesophagus and can lead to pain, inflammation, ulceration, stricture formation, bleeding and cancer. Physiological reflux as measured by high-frequency intraluminal ultrasound probes has been shown to be relatively small in volume. The cross-sectional area of refluxed liquid in healthy subjects averages 7.5mm diameter (16).

Ultrafast CT scanning has shown that on average 15mls of air accompanies a swallowed liquid bolus (13). Repetitive swallowing can therefore cause gas accumulation in the stomach. It has also been shown that the nature of the refluxate into the oesophagus is highly variable. Refluxate can be acidic, non-acidic, liquid or a mixture of liquid and gas. Acidity can be measured intraluminal pH catheters or endoscopically clipped Bravo capsules (17). Deciphering either liquid or gas status can be ascertained by impedance devices (18). The frequency of gastro-oesophageal reflux, which is a normal physiological event, has been shown to be similar between healthy controls and GORD patients. One third of reflux episodes are non-acidic in both groups, with liquid-gas episodes being the most common.

Pure liquid reflux is more acidic in GORD patients (19) and indicates why GORD patients have higher 'DeMeester scores' (20) on 24 hour pH

recordings. This suggests variations in gastric acid secretion or distribution within the stomach. Oesophageal acid clearance is also important. This study did not compare hiatus hernia patients to hernia free subjects. When present together, liquid reflux precedes gas on approximately 40% of occasions, with gas initiating only 25% of episodes (21). Reflux in GORD patients is as likely to occur in the upright posture as in the supine (22), which differs from normal subjects.

Gastric acid secretion increases following a meal. Buffering of the meal contents causes the intragastric pH to rise. In the fasting state, the pH step-up from the fasting acidic stomach to the neutral oesophagus occurs at the SCJ (23). Post-prandially, one would assume that buffering causes the step-up to be lost. However, an unbuffered pocket of acidified gastric juice occurs at the cardia region, with a pH as low as 2 (23). This is likely to be the source of the acidic refluxate. Subtle features can occur at the GOJ in reflux patients, even before the evolution of oesophagitis. The presence of cardiac mucosa, carditis and intestinal metaplasia at this site are frequent. This indicates that the cardia region is not resistant to acid or the other noxious stimuli in the refluxate (24). The gastric contents become hypertonic after a meal. A Barrett's segment is insensitive to acid, but causes symptoms when exposed to hypertonic matter (25).

A Los Angeles group demonstrated that an elevated body mass index resulted in a greater acid exposure of the distal oesophagus in those with reflux symptoms (26). A further retrospective questionnaire of U.S. nurses correlated BMI to symptoms of GORD which varied with changes in BMI over time (27). This contradicts the Swedish data collected in an interview based study. It compared reflux symptoms to patient BMI, without subjective

evidence of oesophageal acid exposure (28). Surgical procedures aimed at aiding obesity affect gastric emptying and as such influence patterns of gastro-oesophageal reflux. Roux-en-Y gastric bypass is efficacious for acid reflux by aiding gastric emptying. Whereas gastric banding is an aggravating factor, possibly by altering food distribution and acid buffering within the stomach (29).

There are several features associated with the integrity of the LOS. The pressure exerted in this high pressure zone is important. There are numerous components to this including the circular muscle of the oesophagus, gastric sling fibres and the diaphragmatic crura. The LOS is an asymmetrical structure with regards to both the pressure exerted and muscle thickness (30;31). Intraluminal ultrasound probes have shown that the circular muscle is the thicker of the 2 layers. The distribution of longitudinal muscle thickness parallels the circular muscle. The maximum pressure recorded at the high pressure zone correlates to the point of maximal muscle thickness (32). In the relaxed state, the LOS is a more symmetrical structure. Its' resting asymmetry probably relates to the mucosal folds and pressure from external structures. Sphincter pressure is a labile phenomenon, even reacting to emotional stress (33).

The length of the LOS, and more importantly, the proportion exposed to intra-abdominal pressure, also has a role. The crural diaphragm is a key component in the anti-reflux barrier (34). Although not directly comparable to disease in humans, the angle of insertion of the oesophagus into the stomach in dogs has been shown to be one of the major factors in preventing reflux oesophagitis (35). As well as the angle of insertion of the oesophagus into the proximal stomach (36), the mucosal folds at the cardia form the final

tight seal of this protective barrier (37). Within these mucosal folds are, as described, a well-defined layer of muscularis mucosae and venous network. Studies have shown some parts of this make-up to be more important than others and some are overlooked entirely. Normally the veins lie deep to the muscularis mucosae. However, the tributaries responsible for variceal formation perforate the muscularis mucosae just above the SCJ (38).

Some investigators believe that the proximal gastric mucosa plays the part of a flap valve (39), which is pushed into position by the increased intragastric pressure during inspiration. A series of *in vivo* experiments with dogs, under anaesthesia, showed that the pressure of water required to cause flow from the oesophagus to the stomach was 15cm. However, the pressure required to cause retrograde flow was 100cm. This gradient was reduced from 100cm to 30cm following severance of the left side of the diaphragmatic loop, which is key to the insertion angle at the cardia (40). A flap-like structure has been seen in humans whilst retroflexing during endoscopy (39). A diminished or absent mucosal flap has been associated with reflux symptoms and oesophagitis (41).

The LOS pressure, contributed to by the factors above, certainly appears to be lower in patients with increasing degrees of oesophagitis. LOS pressure also reduces with an increasing extent of Barrett's oesophagus, partly due to the presence of a hiatus hernia and dissociation of the circular muscle from the diaphragmatic crura. Smokers have lower resting LOS pressures. They seem to suffer from more reflux events, where the LOS is overcome by coughing and also more prolonged LOS relaxations (42). However, there is a wide overlap that exists even amongst healthy volunteers. Some have low pressures and paradoxically, some with

oesophagitis have normal pressure measurements (15). Extrinsic innervation of the human LOS alone does not regulate the resting tone, as demonstrated by surgical vagotomy (43). *In vitro* LOS muscle strips from controls and Barrett's patients exhibit similar basal tone. There is only minimal contraction to substance P in either group with resistance to axonal sodium channel blockade in the Barrett's patients. Neurokinin 2 (NK-2) receptors predominate over NK-1 and NK-3 subtypes (44).

A hiatus hernia always accompanies Barrett's oesophagus. As well as the change in anatomy there comes a movement of the SCJ. In normal individuals the SCJ, identified by endoscopic clipping, is 0.5cm below the diaphragmatic hiatus. Manometry shows that the high pressure zone extends approximately 1.1cm distal to that. In hiatal hernia patients, the GOJ high pressure zone has two segments – the intrinsic LOS and the crura. In non-Barrett's hernia patients the SCJ appears at the lower end of the intrinsic LOS, in an area almost devoid of pressure and abutting onto gastric, acid-secreting mucosa (45). There is good data to show that larger hiatal hernias are associated with weaker, shorter LOS's and impaired oesophageal acid clearance (46). Non-reducing hiatus hernias especially have impaired acid clearance and increased acid exposure. One study of this showed that fewer reflux events occurred. This was probably related to impaired acid clearance and serial reflux events being missed due to no perceivable drop in pH (47).

It is important to keep in mind that the majority of clinically invasive investigations tend to take place in the fasting state, when the GOJ is not being directly 'stressed' or 'tested'. Body position and gastric distension play crucial roles, as both have important physiological implications.

Pressure measurement techniques of the upper GI tract have developed over the last 20 to 30 years. Accurate assessment of individual components of the LOS, intraoesophageal and intragastric pressure (IGP), and their changes with respiration are now possible (48). Analysis of the abdominal portion of the sphincter also shows variation with disease. During respiration there are large changes in intra-thoracic and intra-abdominal pressure. During inspiration a negative pressure exists in the thorax, which one would expect to tend to induce a reflux episode, if it were not for the physical barrier of the LOS. The abdominal length of the sphincter can be calculated with differing accuracy from manometry tracings. The respiratory inversion point (RIP) shows a change in pressure on the tracing, at end expiration, from a positive to a negative deflection (49). The distance from this point to the distal end of the sphincter is the abdominal sphincter length. This length is significantly longer in healthy volunteers than in any patients with lower oesophageal disease. In fact, restoration of the abdominal sphincter has become the feature of some corrective surgeries available for severe reflux disease.

The role of *H.pylori* in GORD patients remains controversial. Gastric colonisation causes chronic gastritis with loss of glands over time, in a majority of patients (50). The rate of progression is slow and dependent on the severity and distribution of the gastritis. Those with maintained acid secretion have gastritis mainly in the antrum and are unlikely to develop atrophy in the corpus. Eradication of *H.pylori* does not alter the dose of proton pump inhibitor required or the pattern of reflux symptoms in GORD patients (51).

1.4 Oesophageal mucosal resistance:

pH recordings from the lower oesophagus will show that a healthy volunteer has a reduced acid exposure in comparison to a reflux patient. The three mechanisms for acid clearance from the oesophageal lumen are gravity, peristalsis and neutralisation of the acid by swallowed saliva and mucosally secreted bicarbonate. Inbuilt defence mechanisms exist to protect the oesophageal mucosa from the physiological phenomenon of gastric acid reflux. Within the oesophageal lumen, and before the epithelium is even reached, there is a mucus layer, along with water and secreted bicarbonate ions. This is more marked within the stomach and duodenum (52). If breached, the tight junctions between the partially keratinised squamous cells slow the passage of H^+ ions into the interstitial space, where buffering takes over. Cell membrane transporter proteins also participate in the epithelial defence systems. If destroyed, new squamous cells can be regenerated, with a 7-day cycle observed in rats (53).

Oesophageal epithelium cell turnover has also been shown to increase in dogs in response to acid exposure and injury (54). A different mechanism, known as 'epithelial restitution' occurs in gastric and duodenal mucosae in response to breachment. In the stomach and duodenum it takes 30-60 minutes and involves the epithelial cells adjacent to the area of injury flattening and migrating over the bare basement membrane, to restore integrity. The most important post-epithelial defence mechanism is the presence of an adequate blood supply to the tissue. It maintains acid-base balance as well as supplying oxygen and nutrients. This is demonstrated as

the blood supply to the oesophagus increases in response to stress caused by luminal acid exposure (55;56).

1.5 LOS function:

Several studies have looked at the effects of food-stuffs on static LOS parameters, in particular resting LOS pressure. Using 240cc intragastric meals, Babka *et al* found no effect on LOS pressure from still water. Both orange and tomato juice individually showed reduction in LOS pressure for a 15-minute period, post-ingestion. Whole milk caused a small reduction in LOS pressure, which was sustained. Non-fat milk showed a significant rise in LOS pressure, 40 minutes post-ingestion (57). Alkali alone had previously been shown to increase LOS pressure (58). The obvious difference here between the 2 milk products was the fat content, indicating that a slower mechanism, probably hormonal, being responsible rather than that of minimal gastric distension. Low-fat chocolate syrup causes an almost dose-response reduction in LOS pressure thought to be mediated by the phosphodiesterase inhibition properties of methylxanthines (57).

Looking at products with lower caffeine loads than chocolate syrup, a set volume of a carbonated cola product was infused into a supine human stomach and compared to a caffeine-free equivalent (59). A drop in pressure, but not significantly different, was seen in both groups. Carbonated water caused a significant drop in LOS pressure compared to a similar volume of still water (59). These studies indicated the importance of gastric distension by gas in reducing resting LOS pressure.

1.6 Oesophageal function:

During peristalsis, external longitudinal muscle contraction precedes that of the circular muscle. Distal propagation speed is normally about 2.5cm/second (60). Physiological changes induced by pregnancy cause a lower wave speed and lower wave amplitude of oesophageal peristalsis (61). Repeated acid exposure to the oesophagus increases its sensitivity and also its contractility (62). Oesophageal contraction in the absence of acid can sometimes mimic heartburn symptoms (63-65). Although acid reflux or oesophageal acid exposure can induce cardiac type pain it does not alter cardiovascular parameters, such as pulse, blood pressure or ECG recordings, in any way (66;66;67).

There is good evidence to show that with an increasing severity of oesophagitis there is an increasing frequency of dysmotility. The resting LOS pressure is lower in patients with oesophagitis and any length of Barrett's compared to controls. The peristaltic wave amplitude of patients with long-segment Barrett's oesophagus (>3cm) is significantly lower than that of controls and those with short-segment Barretts. It is, however, no different from reflux oesophagitis patients. The percentage of non-propagated wet swallows was significantly higher in all 3 groups compared to controls, as was the time of oesophageal pH exposure < 4.0, on 24 pH recordings (68).

Oesophageal contraction in response to intraluminal balloon dilatation occurs primarily distal to the point of stretch. This can be a sustained 'duration response', which causes shortening of the oesophagus. This is caused by contraction of both the longitudinal muscularis externa and muscularis mucosae (69). Electrical stimulation of isolated longitudinal strips

showed this to be of equivalent force in both the muscularis mucosae and muscularis externa. Winship and Zboralske previously commented that they thought this phenomenon would contribute to the oesophageal propulsive force; a mechanism to expel luminal obstruction (70). Secondary peristalsis of the oesophagus aims to clear luminal residue, including refluxed material. It is vagally mediated and sensitive to both atropine and capsaicin. Acidic fluid triggers it at lower levels of distension, and is more resistant to atropine (71).

1.7 Oesophageal shortening:

Changes in the length of the oesophagus have been noted with a shortening in response to acid exposure. It was demonstrated to be a persistent feature in an opossum model, preceding the reduction in LOS pressure and impaired oesophageal motility seen in GORD subjects (72). Chronic oesophageal shortening has even been considered as an early component in the development of a fixed hiatus hernia (73). However, it has been shown that shortening of the oesophagus is less marked with increasing size of hiatus hernia, probably due to less effective contraction of the longitudinal muscles against the phreno-oesophageal attachments (61).

Shortening of the oesophagus by up to 2cm occurs each time we swallow, but is a transient phenomenon. It is observed when assessing the movement of naso-oesophageal pH probes, clearly indicating the reason for positioning them 5cm above the LOS during standard recordings (74). If one swallow quickly follows another, the latter is able to abolish the distal oesophageal contraction and shortening caused by the first swallow. This is a

phenomenon known as deglutitive inhibition (75). *In vitro* studies suggest that pre-treatment with mast cell stabilisers can prevent acid-induced oesophageal shortening, but not that caused by electrical field stimulation (76), alluding to more complex mechanisms being involved via the lumen.

1.8 Gastric motility:

The migrating myoelectric complex (MMC) of the human stomach was described by Stanciu in 1975 (77). It is characterised by bursts of depolarisation that move from the stomach to the ileocaecal valve at regular frequency during the interdigestive period. Individual cycles of activity can last from 1 to 2 hours. One of the major roles of the MMC is to maintain upper gut sterility by sweeping bacteria in the upper bowel downstream and transporting gastric acid from the stomach into the proximal gut. On initiation of a swallow sequence, the gastric fundus undergoes vagally mediated receptive relaxation. Fundal tone is controlled by a balance of both excitatory (cholinergic) and inhibitory (nitrenergic) inputs. As a meal enters the stomach, both tone and phasic contractions in the proximal stomach are inhibited. This is the process referred to as gastric accommodation. This allows the volume of the stomach to increase by 2-3 fold in order to retain the meal, before distributing to the antrum. In the presence of a meal, the antrum is able to separate solids from liquids by a combination of 'sieving' and gastric relaxation (78). Emptying from the stomach is then dependent on phasic pressure waves of the pylorus (79).

Relaxation of the fundus, often as a means of controlling the rate of gastric emptying, is controlled by various physiological events. Antral distension (gastrogastric reflex) is one such event. Acidification of the

duodenal lumen, as well as the presence of lipid and protein (duodenogastric reflex) will also cause this, as will distension of the colon (cologastric reflex). Gastric emptying of liquids is exponential, for those with no nutrient content, and proportional to the intragastric volume. Nutrient containing liquids such as dextrose are emptied slower.

Impaired gastric accommodation in response to a meal has been demonstrated in functional dyspepsia [Tack, J *et al*], showing an association with disease states. Abnormal gastric emptying is also thought to play a role in GORD. Impaired post-prandial gastric motility with an altered distribution of food, leading to variations in proximal gastric distension and acid-buffering which could impact on the disease. In patients with Barrett's oesophagus, duodeno-gastro-oesophageal reflux with bile and pancreatic enzymes (ie: retrograde motility) is considered a key agonist (80). Gastric emptying has been shown to be impaired in 40% of GORD patients with a solid-liquid meal, resulting in larger post-prandial gastric volume (81). Other studies have shown gastric emptying to be normal or rapid in GORD patients (82;83). This could mean that either gastric emptying is irrelevant or that it is important in only a certain subgroup of GORD patients.

1.9 Muscularis mucosae:

Whether impaired motility due to oesophagitis or Barrett's oesophagus is caused by an intrinsic smooth muscle defect or as a result of acid injury to the mucosa, is not known. If the answer lies with smooth muscle dysfunction, then a separate question must be asked. Is the smooth muscle defect within the main muscle bulk of the longitudinal and circular fibres, or is it related to

the often overlooked, muscularis mucosae? Oesophageal clearance requires effective oesophageal peristalsis and delivery of salivary bicarbonate. It takes 7ml of saliva to neutralise 1ml of 0.1M HCl (84). In healthy people, the rate of salivation is the key factor to duration of acid exposure. Impaired ability to empty fluid, as in hiatus hernia patients, can vastly prolong acid clearance (85).

From histological analysis we have seen a marked thickening of the muscularis mucosae at the lower end of the oesophagus (86). This observation has also been seen in felines and guinea pigs (87). In humans its function has not been properly investigated. Thickening of the muscularis mucosae has also been noted in other anatomical sites such as the gastric antrum (88) and rectosigmoid junction (89).

There are reports of double contrast barium studies of the oesophagus in humans revealing both transient and fixed transverse folds in its lower half (90). These have been seen in felines as well, but are a more fixed entity, and restricted to its lower third. In humans the folds are 1-2mm in thickness and cross the entire width of the oesophagus. They have only been seen on 'spot' films and have never been witnessed at GOJ during such studies. These folds are reportedly seen in 1-2% of barium studies, but due to their transient nature, it is thought that the figure could be higher (91). It is certainly a relatively common feature at endoscopy, to induce a corrugated appearance to the oesophagus following air insufflation, perhaps due to reactivity from the muscularis mucosae. This radiological phenomenon has also been seen in barium studies of the gastric antrum (92).

As long ago as 1923, Forsell showed that the mucous membrane of the gastrointestinal tract was freely mobile over the muscularis externa (93).

It's movement being dictated by the muscularis mucosae. Seymour and Meredith also witnessed fine gastric antrum and oesophageal 'rimpling' on double contrast barium studies which were eradicated with increasing distension. The authors attributed these effects to contraction of the muscularis mucosae (94). They also concluded at the time that these folds were of no pathologic significance. During studies to examine the structure of oesophageal varices and the venous network (95) at the GOJ, it was demonstrated that the muscularis mucosae was much thicker at the lower end of the oesophagus. Where the distal mucosa is thrown into longitudinal folds they showed that the muscularis is up to five times thicker at the top of these folds (38). These were in cross-sectional views and the comparative areas were the inter-fold regions.

It is difficult to imagine that this complex arrangement and muscle hypertrophy is not to serve a specific purpose. Using an animal model, isolated strips of muscularis mucosae from the opossum contract in response to electrical field stimulation, giving a sustained response which results in shortening of the oesophagus (69). Although not seen on the barium studies, transverse folds of the lower oesophageal mucosa have been seen in fixed human post-mortem specimens (30;96). These folds are prominent on top of the longitudinal folds already described, and are located at the region of what would relate to the high pressure zone of the LOS. It is possible that during a barium study, and with distension of this area, these folds may be lost until the area returns to it's resting state. The question of embalming artefact should also be considered.

It is therefore highly plausible that the well-defined layer of muscularis mucosae at the top of these folds contracts to cause a 'bunching' of the

overlying mucosa, creating these transverse folds. This would pull the mucosa into the sphincter to form the final part of the seal. Therefore, within the sphincter would be a combination of both longitudinal and transverse folds. The longitudinal folds are formed by heaping of the mucosa by external smooth muscle contraction and the transverse folds by the more superficial muscularis mucosae. There is also some evidence that this thickening of the muscularis may even extend to just below the SCJ (86).

In healthy volunteers a barium study following clipping of the SCJ, showed that in the fasting state, the clips were up to 3cm from the radiological lower border of the LOS. When the gastric fundus was distended the junction was brought to the brink of the stomach proper. This indicated that in a state of distension, the SCJ is likely to be at the interface between the stomach and the oesophagus (97). On occasions during this study, spontaneous migration of the clips, of upto 2cm, toward and away from the stomach would occur. These were not related to gastric emptying or respiratory effort. They were classed as movements of a mobile mucosa propelling itself over the deeper tissues.

With the longitudinal, axial contraction of the muscularis in mind, the issue arises as to whether the SCJ is in fact mobile. Are there any deep fixings of the junction to the underlying tissues? If there is not, and the junction is mobile, this would be very important in relation to diseases at this site. If the z-line were to migrate up the oesophagus, then acid-secreting mucosa below the cardia would have free exposure to the oesophageal mucosa. Vice-versa, if the lower oesophageal mucosa were pulled down into the acid secreting environment of the proximal stomach then acid exposure would also cause subsequent damage.

If a mobile z-line exists, then to keep physiological harmony, it would be important to keep it within the lower oesophageal sphincter. Ideally the transverse corrugated pattern would be best confined to the squamous mucosa portion as otherwise it may promote acid 'trapping'. We are aware that the z-line moves in different states of disease. If there is no change to the lining epithelium then it is important to work out what the cause of this is. Also, if this can be ascertained, then it may indicate whether the movement is the cause or effect of the disease. It may be that there are many temporary minor movements of the SCJ. It could occur in relation to meals and gastric distension, ingestion of active compounds in meals and beverages, or ingestion of pharmacological drugs for non-gastrointestinal ailments.

The opossum's oesophageal structure and function closely relates to that of humans. Acetylcholine causes phasic contractions of its muscularis mucosae via M_1 receptors and substance P a tonic contraction (98). Acetylcholine exists alone and sometimes in the same nerve endings as substance P, albeit in separate granules (99). This itself confirms the complex neurochemical control systems that exist to regulate the function of the muscularis mucosae. Opiate, adenosine, α_2 adrenoceptors and prostaglandin receptors have all been looked for in this tissue but do not appear to be present (100).

The rabbit oesophagus has striated muscle along its length with a muscularis mucosal lining (101). Contracted strips of rabbit oesophageal muscularis mucosae relaxes completely when exposed to 10^{-4} molar sodium nitroprusside. Sodium nitroprusside acts as a NO donor, indicating that NO generating pathways are involved in the control of oesophageal smooth muscle relaxation (102).

1.10 Intestine:

The canine small intestine mirrors closely that of humans, in particular with regards to lumen size and ratio of muscularis mucosae thickness to muscularis externa. This makes it a relatively good model for the effects of neurohumoral and pharmacologic agents in the human intestine. *In vivo*, strips of canine jejunal muscularis mucosae contract in response to intravenous acetylcholine and pentagastrin, as does the muscularis externa. Both layers relax in response to cervical vagi electrical stimulation. They differ in most other responses, with the muscularis mucosae giving relatively tonic responses to intravenous histamine and serotonin compared to the muscularis externa (103).

Increases in small bowel motility in response to CCK, secretin and somatostatin are confined to the muscularis mucosae (104). This work is key, as paradoxical motor effects of the 2 muscle layers had probably been previously misinterpreted or overlooked. Increase in segmenting muscularis mucosae activity associated with atonia of the muscularis propria is seen in the presence of diarrhoea (103), indicating that the muscularis mucosae can dictate disease states. These findings can be reproduced by reserpine, somatostatin and serotonin (103). The muscularis clearly has a vital role in the equilibrium of intestinal function.

These findings backed up earlier work, which showed β adrenergic stimulation lead to contraction of the proximal stomach and small intestine, with relaxation of the antrum. These responses were also attributed to the muscularis mucosae (105-107).

1.11 Transient lower oesophageal sphincter relaxations:

Transient lower oesophageal sphincter relaxations (TLOSRS) were first described in 1980 by Dent *et al.* Initially referred to as 'inappropriate' relaxations of the LOS, TLOSRS are now recognised as the physiological mechanism permitting venting of gas from the stomach. They showed that nocturnal reflux occurred in response to LOS relaxation during arousal from sleep or when fully awake (108). TLOSRS also require selective inhibition of the crural diaphragm (109), which is also seen in vomiting (110) and oesophageal distension (111).

Properties of TLOSRS have been quantified, to classify them separately from swallow-induced LOS relaxation. Dry swallows precede LOS relaxation by a median of 1.4 seconds. The LOS relaxes at a rate of $>1\text{mmHg/sec}$, and the relaxation nadir always occurring within 7 seconds (112). Relaxation duration is always less than 9 seconds. TLOSRS are not associated with swallows either 4 seconds before or 2 seconds after their onset. Relaxation rate is also $>1\text{mmHg/sec}$, with time to complete relaxation of less than 10 seconds. Nadir LOS pressure is $\leq 2\text{mmHg}$. Mean duration of LOS relaxation with swallowing is approximately 4.5 seconds compared to 18 seconds for TLOSRS. TLOSRS also appear to be associated with a prominent LOS after-contraction (112).

A consequence of TLOSRS is often the reflux of gastric contents during 'common cavity' events. Common cavity events are not exclusively related to TLOSRS, with almost 40-60% occurring during loss of sphincter tone by other causes, but not necessarily associated with reflux (113). In healthy subjects TLOSRS and gas venting are suppressed in the supine

position (114) and this has been confirmed to be the case in GORD patients as well (115). In the upright posture TLOSRS cause gas or liquid reflux in 70% of cases in healthy volunteers, but the handling of the refluxate varies in GORD patients as described earlier.

A Nissen fundoplication increases the mechanical rigidity of the high pressure zone, the total high pressure zone length and the abdominal length of the LOS. It also helps to maintain the high pressure zone (HPZ) pressure and length in response to gastric distension and a rise in intragastric pressure (116;117). It appears to be useful in patients with early disease and abnormal LOS features, and also in patients with early disease but mechanically normal sphincters. Due to the maintenance of the HPZ length, the fundoplication procedure reduces the amount of post-prandial TLOSRS (118;119). The degree of gastric distension is clearly an important factor in sphincter function. The length of sphincter improves the mucosal seal by decreasing the importance of gastric wall tension. Less compelling evidence of TLOSRS triggering suggests that stretch receptors of the cardia are more important than tension receptors (120).

It has been shown that TLOSRS are responsible for most reflux events. HPZ parameters have been measured before and after the fundoplication procedure. As stated, surgical procedures involving a 'wrap' around the proximal stomach and LOS region can improve the anti-reflux barrier of the LOS by improving the maintenance of HPZ length in response to gastric distension. In the early stages of disease, the lower portion of the sphincter is inclined to be lost at lower gastric distension pressures, but with maintenance of resting LOS pressure. At the more severe end of the disease spectrum, both sphincter length and resting LOS pressure are reduced

permanently. Complete fundoplication impairs the ability to belch, also reducing the number of common cavity events. With this comes moderate discomfort and the potential limitations of the therapy (113). Although resting LOS pressure is not always effected by fundoplication, a significant increase in nadir pressure during swallow-induced relaxation occurs (121).

Gastric distension itself is a potent stimulus for triggering TLOSRS. Gastric accommodation allows for an increase in intragastric volume as previously described. There comes a point when the forces exerted on the proximal stomach are such that stimulation of receptors here are sufficient to trigger a TLOSRS. In the presence of a hiatus hernia the anatomy suggests that these receptors may be under different stresses than when a meal is in the main body of the stomach. In another study, Dodds *et al* showed TLOSRS to be the main cause (94%) of physiological reflux in healthy volunteers, compared to reflux patients (65%) (122). In reflux patients, rises in intra-abdominal pressure and spontaneous free reflux due to sphincter incompetence constitute the other causes, with approximately equal frequencies.

Kahrilas *et al* showed that a 4mmHg rise in IGP was needed to trigger a TLOSRS (123). A shortening of the distal end (abdominal portion) of the LOS was also seen. A static, non-anchored, multi-lumen manometry catheter was used to assess this. These findings are consistent with the IGP surpassing the pressure of the lower end of the LOS. Using an inflatable bag, Holloway *et al* had previously shown that the rise in fundal pressure required to trigger a TLOSRS was in the region of 2-6mmHg (124). LOS topography has shown that the distal region of the LOS distended by a rise in IGP has an intrinsic tone of approximately 3mmHg (125). This is the 'tail' region of LOS pressure,

which is seen on both slow-motorised and vector volume pull-throughs. Overcoming this closure pressure appears to be a key step in initiating the cascade for TLOSRS to occur. Compliance in the fasting state and also relaxation of the proximal stomach shortly after a meal are not altered in reflux disease (126). Gastric tone reduces in both healthy subjects and GORD patients following a meal. It takes almost 90 minutes for a healthy subjects' gastric tone to return, whereas tone often remains significantly impaired in GORD patients (127). Penagini *et al* showed that GORD patients have a larger proximal gastric volume post-prandially. This reflects delayed recovery of proximal gastric tone, highlighting a potential cause for increased TLOSRS frequency (126).

A 'program generator' area in the brainstem is thought to play a role in controlling TLOSRS. This area contains GABAergic neurones which act in an inhibitory fashion. Baclofen at a dose of 40mgs, which is a GABA agonist, causes a rise in resting LOS pressure and a reduction in both post-meal TLOSRS and reflux events, in healthy volunteers. However, degrees of oesophageal acid exposure and clearance conflict in different studies. (128-130). Similar effects in GORD patients, in bedside studies have been seen, with no clear indication for the role of GABA-B agonists in GORD (131).

Richter described TLOSRS as a vagally mediated reflex, in which gastric distension plays an obvious role (132). TLOSRS have been abolished in dogs following vagal blockade (133). Atropine reduces LOS tone and inhibits TLOSRS in healthy volunteers (134). In canines, atropine at high doses ($381 \pm 0.13 \mu\text{g/kg}$) can abolish all components of the anti-reflux barrier and at lower doses can reliably produce gastro-oesophageal reflux (135).

Various set-ups have been used to trigger TLOSRS. Kahrilas' group used a continuous air infusion just distal to the GOJ as their stimulus. In their hiatus hernia patients, this may have been above the diaphragm. Air-trapping within the hiatal sac was excluded as no pressure gradient was seen between this area and the gastric body. A greater frequency of TLOSRS was seen in hiatus hernia patients and particularly those with a shorter LOS (123). With the absence of the gastric sling fibres at the lower end of the LOS, it is almost in a state of partial distension, even in the fasting state. Thus, for each unit increase in IGP seen, there was a greater effect on the LOS. The increased frequency of TLOSRS was proportional to the hernia size, with no altered IGP threshold (136).

Due to their neurally mediated mechanism, and as previously described, TLOSRS can be interfered with by pharmacological means. A pharmacological reduction in TLOSRS, such as that caused by morphine, has been shown to cause a reduction in the frequency of gastro-oesophageal reflux events in both healthy volunteers and GORD patients (134;137-139). The vagal triggering of TLOSRS is a reflex mediated via the brainstem. Cooling of the vagus nerve abolishes TLOSRS in canines as does vagal transection (136). The compound LNMA inhibits NO synthesis by blocking nitric oxide synthase and has been found to inhibit gastric distension and post-prandial increases in TLOSRS. This implies that neuronal NO controls gastric relaxation and is involved in TLOSRS. No obvious effect of this compound on resting LOS tone has been observed (140;141).

Loxiglumide is a CCK-A receptor antagonist. CCK acts on CCK-A receptors in the proximal stomach to decrease TLOSRS induced by gastric distension. At a dose of 10mg/kg/hr, loxiglumide significantly reduces the

number of TLOSRS and common cavity events in healthy volunteers, without interfering with swallow-induced LOS relaxation (137). This is true for both gastric distension with air and also following an ingested meal or duodenal meal infusion (142). CCK triggers TLOSRS via CCK-A receptors in canines, as does endogenous production of NO (143).

Intraduodenal fat appears to cause a reduction in LOS pressure in healthy subjects but not those with GORD. It suggests that the reduction is equivalent to the inherent tone already lost by GORD patients. It also causes more acidic reflux events during TLOSRS in GORD patients, with no change in TLOSRS frequency (144). Although commonly associated with reflux disease, the majority of studies have shown that meals with a higher fat content do not increase oesophageal acid exposure in healthy volunteers or the already altered pattern in GORD subjects, compared to a balanced fat meal (24%) (145). One study showed an increased oesophageal acid exposure after a high fat meal, but only in the supine position (146). Once again, TLOSRS frequency was not influenced by a high fat load (147).

Some endoscopic procedures for GORD have failed to deliver improvements over existing treatments. The 'Stretta' procedure improved GORD symptoms and quality of life scores, but did not decrease oesophageal acid exposure or medication use at 6 months. The Stretta procedure involves exposing the GOJ to radio-frequency energy in an attempt to either scar the GOJ tissues and/or cause damage to those nerves which trigger pathways involved in TLOSRS and reflux events (148;149).

1.12 Cardia compliance:

There is good evidence that dilatation of the cardia, whether it be by gastric distension or by pharmacological means, predisposes to disruption of integrity of the LOS and consequences of GORD (150). An endoscopic, laser-induced scar, through to the muscularis propria, placed circumferentially around the cardia has been shown to increase the yield pressure of the LOS in response to increased IGP (151), probably due to a 'stiffening' effect on the cardia region.

Reduction in compliance of the gastric cardia is part of the theory behind the success of endoscopic full-thickness plication. Acid reflux is reduced by decreasing the frequency of TLOSRS, but also by reforming the valve-like system at the GOJ (152). It is not surprising that the compliance of the LOS is different in healthy subjects compared to hiatus hernia patients. However, functional behaviour of the GOJ also differs in those GORD patients without a hiatus hernia. Distension of the LOS region and specifically the cardia is more likely to trigger TLOSRS at much lower pressure rises (153).

1.13 Physiology & pharmacology:

High doses of the calcium channel antagonist diltiazem, when given intravenously, do not affect the contractility of the healthy human oesophagus *in vivo* (154), although sublingual nifedipine does in spastic oesophageal conditions (155).

Cisapride was routinely used as an upper gastrointestinal pro-kinetic, until cardiac interaction caused its withdrawal from mainstream clinical

practice. Its mechanism of action is not completely clear. It has 5HT₄ receptor specificity which could potentially stimulate acetylcholine release from post-ganglionic neurones in the myenteric plexus (156). At therapeutic doses in GORD patients, no obvious effects on peristaltic amplitude, duration of contraction, propagation speed or oesophageal acid clearance were seen. Resting LOS tone and post-prandial TLOSR frequency were unaltered in response to gaseous gastric distension. With the effect of gastric emptying on GORD being unclear, this study showed that cisapride's perceived benefits did not appear to alter any of the key components associated with the dysmotility of GORD (157).

Ambulatory oesophageal pH recordings in GORD patients, post-baclofen 40mg po, showed it to cause a reduction in oesophageal acid exposure in the 4 hours after dosing only, and a reduction in belching, presumably linked to TLOSR suppression (139;158).

1.14 Nitrate:

The physiological effects of dietary nitrate on the upper GI tract are still not clear and demand further work (159). Nitrate ingestion has previously been implicated in carcinogenesis (160;161). Nitrate consumed from the diet undergoes an entero-salivary circulation. Over 98% is absorbed in the stomach and proximal small bowel. It is then actively transported to the salivary glands to achieve higher concentrations (mean 252 $\mu\text{mol/l}$, range 32-600 $\mu\text{mol/l}$) than that found in the blood stream (mean 95 $\mu\text{mol/l}$, range 32-152 $\mu\text{mol/l}$) (162). Nitrate is secreted into the mouth where it is converted to nitrite by bacteria on the dorsum of the tongue and the buccal mucosa. This

step is accomplished by the bacterial enzyme, nitrate reductase (163). Salivary nitrite is then swallowed, passing down the oesophagus by peristalsis, reaching the LOS and cardia region. The pH within the upper GI tract varies with its location. The pH is often between 6.4-8.0 in the mouth depending on salivary flow-rate, ingested food and mouth sterility (164). Within the lumen of the oesophagus the pH is neutral (6.5-7.0). However, in a healthy acid secreting stomach, the pH is approximately 1.0-1.5 in the fasted state. In the absence of luminal anti-oxidants the salivary nitrite will immediately be converted to nitrous acid, of which 1% will exist as dissolved NO (165). In the presence of luminal anti-oxidants such as ascorbic acid, which is secreted by the mucosa of a healthy stomach and also abundant in foodstuffs, the nitrite will be immediately converted to NO in a 1 to 1 molar ratio. Thiocyanate is secreted into saliva and is swallowed along with the nitrite. It then catalyses its conversion to NO within the stomach (166).

Ingestion of 2 millimoles of nitrate causes salivary nitrite levels to rise to millimolar concentrations and when this is swallowed it causes the level of gastric ascorbic acid to decrease, due to its oxidation (167). Work by Casselbrant's group in 2002 reconfirmed that luminal oesophageal NO required saliva rich in nitrite. This accounts for 95% of the production. The other 5% is formed by L-arginine metabolism in the epithelium of the oesophageal mucosa (168). The NO concentration has also been shown to increase in the oesophageal lumen during reflux events (166), but under normal conditions, its generation is maximal at the SCJ (162).

N-nitroso compounds are formed in the proximal stomach when the nitrous acid, in the absence of anti-oxidants, react with secondary amines present in food. These are potentially carcinogenic due to their ability to

alkylate DNA. Thiol compounds present in food can also react with the nitrous acid to form nitrosothiols which behave as long-acting NO donors. Therefore, depending on the availability of ascorbic acid in gastric juice at the proximal stomach, various physiologically active compounds may exist. For some time now, colonisation of the achlorhydric stomach by denitrifying bacteria has been considered as a major precursor for gastric carcinogenesis (169). The bacteria can potentially produce nitroso-compounds from swallowed nitrite (170). The incidence of atrophic gastritis in the Western world is decreasing and with it gastric cancer.

The duration taken for ingested nitrate to appear as oral nitrite is approximately 25 to 30 minutes, with a peak at 1 to 2 hours. Some work was performed to look at the effects of several proprietary mouthwashes and anti-plaque toothpastes on the reduction of oral nitrate. Using a chlorhexidine containing mouthwash (0.2%) reduced the mean percentage of salivary nitrite from $16.1 \pm 6.2\%$ to $0.9 \pm 0.8\%$. There was no effect from 0.03% triclosan or amyloglucosidase mouthwashes. 0.3% triclosan/0.75% zinc citrate 'anti-plaque' toothpaste also failed to have any impact. The use of chewing gum caused a rise in the pH of the oral cavity from 6.8 to 7.3, associated with a significant rise in the conversion of nitrate from 12.2 ± 10.7 mg/l to 31.7 ± 21.3 mg/l. This is most probably due to the pH being closer to optimal pH of 8 for the nitrate reductase enzyme. Previously thought to play only an anti-bacterial role (171), luminal NO has since been shown to modulate mucosal functions such as gastrin release (172). It is plausible that local luminal NO in the LOS may affect afferent receptors and by the reflex arcs described, in turn modulate LOS function.

1.15 Nitric oxide:

Endogenous NO release was discovered to be the 'endothelium derived relaxing factor' responsible for vascular smooth muscle relaxation (173;174). Binding of NO to soluble guanylate cyclase in the cytoplasm of the smooth muscle cell, increases its activity. Conversion of guanylyl triphosphate (GTP) to cyclic-guanlyl mono-phosphate (cGMP) alters Ca^{2+} -Calmodulin binding affinity, to reduce tone (175) – see Figure 1.2.

Cyclic GMP is metabolised to the inactive form GMP by phosphodiesterase V. Sildenafil is a phosphodiesterase subtype V inhibitor thereby enhancing the end action of NO (176;177). Sildenafil causes a significant reduction in human LOS tone *in vivo*, with the potential to abolish lower oesophageal peristaltic contractions. At a moderate level of action, sildenafil impairs oesophageal acid clearance in the supine position. Only if the effect is very severe does impaired oesophageal motility impact on oesophageal acid clearance in the upright position, with a healthy human oesophagus (178). Sildenafil is also effective in treating patients with hypertensive oesophageal or LOS conditions (179), but side effects are limiting.

Thirty minutes of acid exposure into the human oesophagus causes an increase in luminal NO production, which is more marked in GORD subjects (168). It does not appear attributable to increased iNOS activity (180). The raised levels of NO occur even without the presence of swallowed saliva (168;180). Endogenous NO influences pyloric tone (181) and also increases gastric mucosal blood flow in response to gastric acid secretion (182). Non-adrenergic non-cholinergic nerves release NO in response to

increases in gastric pressure. In the guinea pig, this is by 2 distinct neuronal pathways. One is a local reflex arc causing NO release, and the other is a ganglion nicotinic controlled mechanism (183). NO donors, such as glyceryl trinitrate (GTN) have been shown to reduce pyloric pressure waves, wave amplitude and frequency in humans (184). Sublingual GTN has also been shown to reduce gastric emptying of a liquid meal in healthy humans (185).

In the dog antrum CCK-B receptors initiate contraction, whereas they initiate NO release and relaxation in the intestine. This study reaffirmed that in targeting a particular receptor pharmacologically, it will not always have a simple physiological effect (186). In contradiction with standard effects, one group has shown a mild excitatory component of NO in the feline oesophageal body (187). This illustrates the need to appreciate that each animal model varies, even when considering well-known neurotransmitters with recognised mechanisms of action. The site of NO production varies, depending on the activity of the generating enzyme. In porcine small bowel iNOS is present in the epithelium, with the endothelial and neuronal subtypes located in deeper layers of the jejunal wall (188).

1.16 Other potential mechanisms of nitric oxide other than cyclic GMP & soluble guanylate cyclase:

The NO molecule is a free radical. In biology the principal reactants are transition metals, in particular iron (189). NO has been shown to bind to the iron binding site of haem in a reversible manner (190). NO controls intracellular oxygen consumption by inhibiting cytochrome c oxidase, which is an electron acceptor in the mitochondrial electron transport chain. At high

concentrations NO binds to the ferrous ion at the binding site in a reversible fashion, and to oxidised iron at lower concentrations. The O₂ binding site contains both iron and copper.

It is a dynamic system with NO playing an important role in cell respiration and perhaps mechanisms of cell survival and death (191). The O₂ tension *in vivo* in mitochondria is probably in the region of 30-50µm and <10µm in highly active tissue, or tissue with a poor blood supply (192). Of note, for luminal NO to reach these concentrations in cellular mitochondria, the diffusion gradient would need to be so high that smooth muscle effects, are likely to be inevitable.

Nitric oxide inhibition of cytochrome oxidase may also be involved in the cytotoxicity of NO and may cause increased oxygen radical production by the mitochondria. This may lead to the generation of peroxynitrite (NO and superoxide anion: O₂⁻ (O₂ minus an electron)), which can damage mitochondria, and is a potential contributor in a variety of pathologies (193). Nanomolar levels of NO inhibit cytochrome oxidase, raising the Km for cellular respiration (194). Peroxynitrite, as well as micromolar NO can irreversibly inhibit this enzyme, with potentially dramatic effects in tissues with low oxygen concentrations (195).

Nitric oxide can come out of solution to form gas. Its activity at this point is greatly reduced. Whilst in solution it can react with thiol groups to form N-nitrosothiols, which are relatively stable at 37°C. Their haemodynamic effects, when delivered intravenously, mimic those of nitroprusside and Glyceryl Trinitrate (196). Similar effects have been seen in rat gastric tissue (197). Thiols exist *in vivo* and are ingested in large quantities. Nitrosothiols,

or thionitrites as they are also known, are synthesised when parent thiols react with nitrous acid (198).

Two nitrosothiols of biological relevance have been isolated and characterised. S-nitroso-N-acetylpenicillamine (SNAP) and S-nitrosoglutathione (GSNO). The biological half-life of the latter is 10 hours at 37°C. These compounds are able to bypass normal mechanisms of NOS activity and still deliver a source of NO to tissues (199-201). SNAP and NO cause an increase in cGMP activity and have been shown to depress GABA_A receptor function in retinal cells *in vitro* (202).

Nitric oxide generated in the body will react with O₂ and preferentially form S-nitrosothiol adducts rather than nitrate (203). This is a process that is likely to occur in the region adjacent to the epithelium of the cardia. Glutathione concentration in cells is approximately 10mM and in plasma 5-25µM (204). S-nitrosothiol circulates in plasma and its concentration is approximately 20nM (205). Plasma ascorbic acid concentrations are generally in the range of 40-80µM. Ascorbic acid, in the presence of copper, at concentrations >0.1mM causes dissociation of NO from S-nitrosothiols (206).

1.17 Research aims:

The aim of this series of experiments is to assess the potential effects of luminal nitric oxide in the upper gastro-intestinal tract, by modelling the acidification process of luminal salivary nitrite, generated from ingested nitrate, in humans. A sensitive system needs to be developed, from the currently available equipment, in order to collect useful data that may

enhance our ability to ascertain physiological effects that may occur in both healthy subjects and those with upper gastro-intestinal disease.

Figure 1.1: Histological appearance of the mucosa at the human gastro-oesophageal junction.

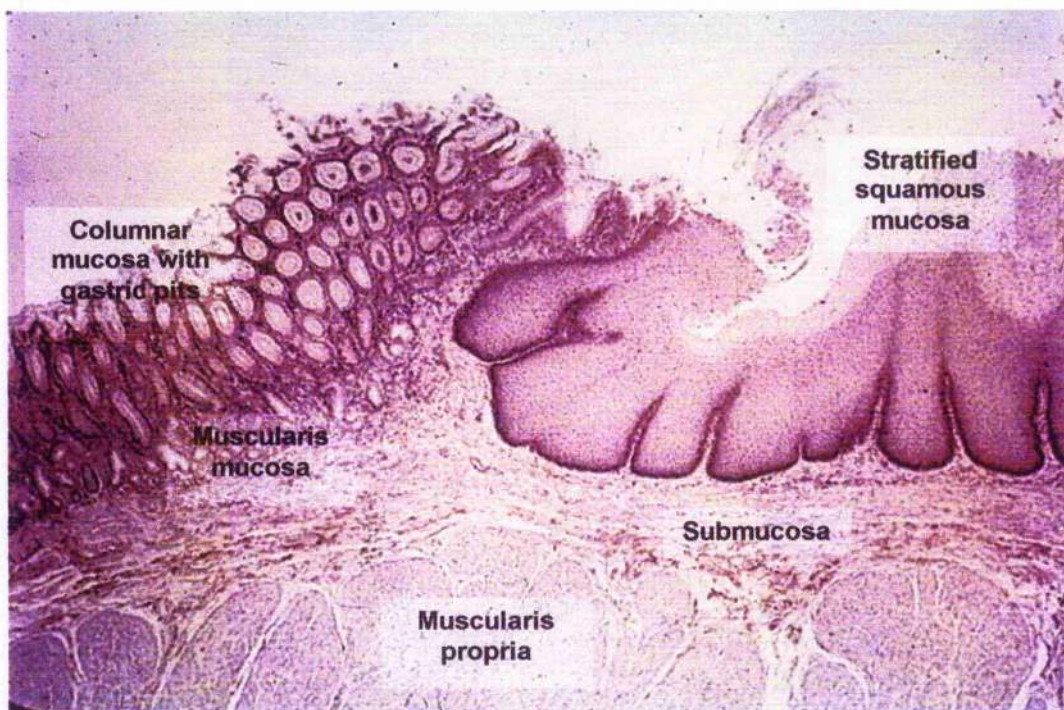
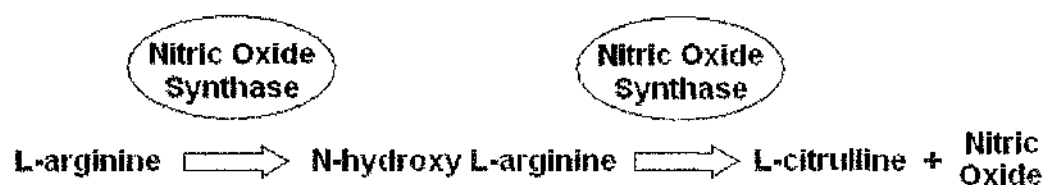


Figure 1.2: Neuronal nitric oxide generation is normally formed intracellularly (A), and can then diffuse across cell membranes and into neighbouring cells to activate soluble guanylate cyclase (B).

A:



B:

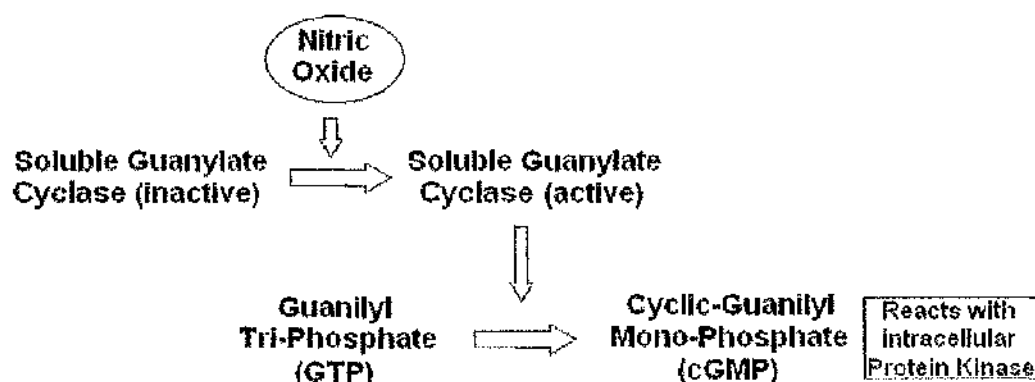
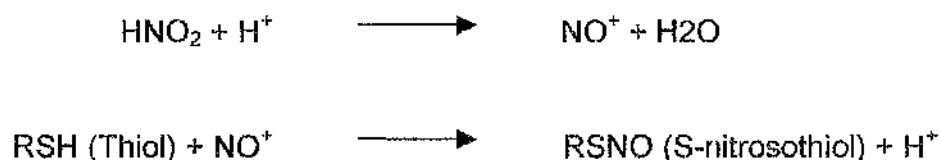


Figure 1.3: Reaction occurring in the generation of nitrosothiols.



[Hogg N *Free radical Biology & Medicine* 2000;28(10):1478-1486]

A novel system for delivering nitric oxide to the lumen of the proximal stomach, adjacent to the cardiac mucosa, with concurrent pressure analysis.

Chapter 2

Chapter aims:

This chapter describes the design process and testing of the manometry catheters and perfusion set-up that would be used for both our fasting and post-meal protocols. We would be using a unique NO generating solution perfused through the manometry catheter. The justification for this solution and laboratory assessment of the NO dose generated for each study is described. Evidence of a biological effect of the NO solution on human skin is shown.

2.1 Abstract:

2.1.1 Background:

The incidence of cancers at the GOJ and lower oesophagus is increasing (207). Recent work has shown that NO is generated at high concentrations in the lumen of the upper gastro-intestinal tract, and that this is maximal at the interface of the GOJ (162). Nitric oxide from this source is derived from dietary nitrates and not the L-arginine route of neuronal generation (208). Neuronal NO is key in the relaxation process of the LOS (196). An important issue to explore is whether NO generated within the lumen can affect LOS pressure and hence predispose to acid reflux, which is a major risk factor for cancers at this site (209).

2.1.2 Aim:

To design systems that can record manometry of the oesophageal body and LOS, whilst delivering test solutions to the lumen of the cardia region. This would need to be viable in both a fasting and post-prandial state.

2.1.3 Method:

A standard water perfused manometry system was adapted to record pressure and also deliver a test solution. These systems were validated against standard clinical apparatus. Selected manometry channels, normally perfused with water, were perfused with a NO generating solution from a syringe driver. Profiles of NO concentration were assessed using various *in vivo* and *in vitro* techniques.

2.1.4 Results:

We have designed separate systems that accurately deliver a known volume and concentration of NO solution to the luminal surface of the gastric cardia. It is still possible to record pressure accurately and detect LOS relaxation. This chapter describes system testing with validation of results.

2.2 Introduction:

Oesophageal manometry has long been used as a tool for investigating patients with difficult reflux disease and oesophageal symptoms. Several studies have looked at individual dietary components to assess their effects on LOS parameters, such as maximal pressure and total sphincter length (57;59). Motility studies of the upper GI tract are commonly undertaken in both the diagnostic and research settings. In order to investigate the oesophageal body and the 'sphincters' at both its proximal and distal ends, the two choices available to the clinician are to use either a solid-state catheter or a water-perfused manometry system for the recording and analysis of pressure.

Sterilisation procedures for both types of catheters are similar and the initial cost of a water perfusion hydraulic pump system may be offset by the high costs of a solid-state catheter. In clinical practice, protocols with standardly available catheters are used widely for both symptom investigation and pre-operative assessment. When used as a research tool, standard manometry catheters can be used, but individual designs can be accommodated by most manufacturers, in order to address a specific question.

Working in a Research Unit with a focus on the luminal generation of NO at the GOJ, we were keen to explore its effects on both motility and acid reflux. About 25% of nitrates ingested in the diet are absorbed from the small bowel and subsequently taken up and concentrated in the salivary glands (210). When secreted into the mouth, buccal bacteria converts approximately 25% of the nitrate into nitrite (211) (Figure 2.1). This nitrite is swallowed and

when it encounters gastric acid in the presence of ascorbic acid, which is actively secreted by the stomach, it could theoretically be entirely converted to NO in a 1:1 molar ratio (162;212) (Figure 2.2).

One particular study has looked at the effects of dietary nitrates on oesophageal motor function. Bove *et al* supplemented patients with oral nitrates (159), but found no significant effect on LOS function in healthy volunteers or GORD patients. In their study they measured salivary nitrate when fasted for manometric assessment. Their healthy volunteer's values (mean $7.1\mu\text{mol/l} \pm \text{SD } 8.4\mu\text{mol/l}$) are lower than we have recorded in the laboratory (mean $252\mu\text{mol/l}$, range $32\text{-}600\mu\text{mol/l}$). Certainly the issue of patient compliance in taking nitrate supplementation was raised. The entero-salivary circulation of ingested nitrates takes approximately 30 minutes and tails off thereafter. Therefore, it can be assumed that the levels measured at the time of the manometry study are not those maximally achieved.

They also found no difference in the LOS pressure, but the absolute pressure values did appear to be rather on the low side, especially for healthy volunteers and even for patients without a hiatus hernia or Barrett's oesophagus (15). There was also no measure of the LOS pressure after distension and whether this varied between the healthy volunteers and the GORD subclasses. One observation made of the 24 hour intragastric pH recordings was that the total time that intra-gastric pH was below 3 was less in the GORD group than the healthy volunteers. Although not reaching statistical significance, compliance of abstinence from anti-secretory medication must also be brought into question. These reflections alone

highlight the difficulties experienced in trying to detect what is expected to be a subtle physiological change.

We were keen to measure LOS parameters in the presence of high concentrations of NO. We have measured luminal NO in the upper GI tract in the region of 60 micromoles per litre. We felt that these concentrations could actually be surpassed as these levels measured were at the upper limit of our customized probe's sensitivity (162). We decided to see if much higher concentrations of NO would show a difference on sphincter function, primarily pressure, and initially in the fasting state. The challenge was to measure the pressure profile of the LOS whilst delivering controlled amounts of NO to the mucosa. In order to achieve this we decided to design a delivery system of known NO concentration rather than rely on unreliable and variable salivary values.

2.3 Fasting study:

2.3.1 System design and method:

A standard 8-lumen water-perfused manometry catheter commonly used for fasting oesophageal analysis has 4 radial channels at the same level orientated at 90° to each other. The remaining 4 channels are placed at 5cm increments proximally, in a helical arrangement, up to 20cm proximal from the 4 'LOS' channels. Each lumen is perfused individually, by a column of water, from a 'common' manometry chamber. The water in this chamber is under constant pressure from compressed air and the water is pumped through capillary tubing before passing through 8 pressure transducers. These then connect onto the 8 ports of the manometry catheter. Pressure registered to the transducers is relayed to a Personal Computer and processed by a software package; in our case, Polygram NetTM (Medtronic, Denmark). This relay of information occurs by the back pressure of the column of water pressing on a sensing chip which sits in a hollow covered by a sealant gel. Back pressure implies that the hole is occluded and therefore in contact with the mucosa. At the GOJ, using these flowing columns of fluid as a vehicle, this would give a system for NO delivery that would have direct mucosal contact, similar to that under normal physiological conditions.

Our delivery solution would, therefore, have to mimic the physiological components present in the upper GI tract. These include salivary nitrite in the form of Sodium Nitrite salt (Sigma, UK) at a concentration of 1.6mM. Basal salivary nitrite levels are variable (mean 36µmol/l: range 19-153µmol/l), but after a standard 2 millimole nitrate meal they can rise substantially (mean 252µmol/l: range 32-600µmol/l) (162). Taking into account the flow rate of

saliva, and therefore the total amount of nitrite delivered, we are dealing with equivalent post-prandial levels in our protocol. Thiocyanate, a normal component of saliva, which aids catalysis of the reaction, also present as the sodium salt (Sigma, UK), 1mM, is within the normal physiological range. Ascorbic Acid (Sigma, UK) would be in solution at 5mM, which is higher than occurring naturally, but in total will equate to 250 μ moles. Within the enclosed syringe, it is present in excess, (80 μ moles in the syringe), compared to the nitrite and therefore will encourage the reaction as described. This will be described in more detail, for the fasting study, in 2.6.1.

Finally, as this is a pH dependent reaction, hydrochloric acid at a pH of 1.8 was added, to mimic the environment of a healthy acid-secreting stomach. A summary of the solution contents can be seen in Table 2.1. The delivery of this solution raised two main issues. Firstly, where in the upper GI tract we intended to run this solution, and also, how many of the 8 standard manometry channels we wished it to pass through? If we had wanted to use our solution through all 8 of the available channels then simply placing our solution, as described above, into the manometry pressure chamber would have been an option. However, the hydraulic water chamber supplied with our Mui Scientific system (Model PIP-4-8, Mui Scientific, Canada) is equipped with brass fittings, which would corrode in the presence of hydrochloric acid at our intended pH. This would also deliver NO along the length of both the catheter and the oesophagus.

The chemistry of swallowed salivary nitrite is a pH-dependent reaction and its conversion to NO occurs as soon as it meets the acid environment of the stomach. To mimic the physiological state as close as possible, and aid

catheter placement, it benefits us to know where this pH gradient occurs. Fletcher *et al* (23) placed radiolucent clips endoscopically at the SCJ and performed slow pH pull-throughs with antimony crystal pH catheters. This showed that, in the fasting state, the pH step-up point from the acidity of the gastric cavity to the neutrality of the oesophageal lumen occurs just distal to the SCJ. With the aid of manometry studies Csendes *et al* also showed that in a healthy population the SCJ is positioned almost centrally within the high pressure zone of the LOS (15). This implies that the nitrite chemistry takes place adjacent to the mucosa in the lower portion of the LOS, an area referred to as the gastric cardia.

This is the area on which we concentrated. We decided to limit the delivery of NO to the four distal radial channels. These could be located in the gastric portion of the LOS (ie cardia), and left for a period of infusion - Figure 2.3. Positioning of the catheter was achieved by performing a pH pull-through and then relocating the manometry catheter to the pH step-up level. The problem then arises with how to supply the NO to these channels and still measure pressures from the transducers.

2.3.2 Alternative designs and flaws:

The initial setup to split the inflow from the syringe driver to the 4 transducers gave variable and erratic pressure recordings. The syringe supply was divided into 4 using a 'traffic light' junction. Each of the 4 outflows came off in turn to feed their respective transducer. The reason for the variable flow became clear when blue food dye was added to the syringe's solution. This revealed a preferential and greater flow-rate through the first outlet, with increasingly reduced flow through the subsequent 3 channels,

manifested by a more dilute colour - Figure 2.4. Splitting a common inflow supply, in the form of the syringe, was perceived as the correct way forward, and a further variation of this theme was devised.

We decided that the 'oesophageal' channels would be supplied from the standard water chamber and the water flow to the other four channels switched off using the array of stopcocks on the compressor unit. An external syringe driver (Perfusor^R, B.Braun Medical Ltd, UK) was used to deliver standard perfusing water and also a NO generating solution to the remaining 4 channels. It's occlusion alarm pressure was 300-600mmHg. This syringe driver used pump-specific 50ml syringes (Original-Perfusor-Spritze OPS 50ml) with a leur lock connection, and a variable flow-rate of 0.1 – 99.9 ml/hr. A four-way dividing loom was designed to supply each of the 4 LOS transducers directly to their lower poles - Figure 2.5. A short section of tubing (Female-Female Luer, R809, Avon Medicals, UK) took the outflow from the syringe driver via a three-way stopcock (876.00, Vygon, UK). A Y-connector (884.04, Vygon, UK) divided this flow equally, each arm of which then joined a further R809 section.

Each of these two sides would supply two of the pressure transducers. A further Y-connector (884.04) on each side lead to a lectro-cath (1155.01, Vygon, UK), totalling four in all. Each lectro-cath, by means of a male luer connection, was then secured onto the inferior pole (inflow) of a pressure transducer. The individual components of this assembly were secured together using an epoxy resin based adhesive (Araldite Rapid^R, Bostik). The 50ml syringe of the syringe driver was connected to the 3-way stopcock with a 100cm luer-lock Lectro-Cath (1155.10, Vygon, UK).

2.3.3 Design performance:

It was important that we could rely upon this system to accurately record pressure changes at the LOS. Rigorous testing took place, initially to see how these 4 channels compared to standardly perfused channels, when they were being calibrated prior to a study. For water perfused manometry systems, a two-point calibration is usually performed. Low and High values, 0 and +680mm apart vertically, are used which is equivalent to a pressure difference of 50mmHg. The 4 radial channels were tested on the bench top in 2 ways. Firstly, pressure differences were measured with these channels sitting in a measuring cylinder and water added to increase the pressure in 5mmHg increments, up to 25mmHg. Secondly, the catheter was placed in the same measuring cylinder and a similar column of water added in a rapid fashion, to look at the response time to pressure changes. Regular pressure transducers are perfused by water passing through capillary tubing. This is accepted as the gold-standard for pressure measurement with water perfused manometry. For both the 5mmHg-equivalent increments and the fast-fill procedure there was less than $\pm 2\%$ difference between the two manometry methods. With this knowledge we progressed onto *in vivo* studies.

Our study would involve periods where the catheter was static and also periods when we were required to move it in a controlled fashion. Moving the catheter recording channels proximally from the stomach to the oesophagus is important to collect data on the LOS characteristics. Specifically data related to the pressure, length, and especially these factors relative to the position within the sphincter. In most out-patient set-ups, the station pull-through technique is used to accomplish this. This involves the

manometry recording channels starting in the stomach and being withdrawn proximally a designated distance (0.5 to 1cm) from the patient's nostril every 30 to 60 seconds. A marker is routinely added to the computer software and data can be interpreted for these set places. This technique serves well for clinical practice, but when assessing pressure changes on a trace, there is the possibility that a higher pressure may have occurred between the two data markers. Also, the proximal or distal end of the sphincter may not have been picked up accurately. If using 1cm data collections the sphincter border measurement could be inaccurate by up to 9mm. It is possible that this could occur at both ends of the LOS and the errors could then be compounded into the calculation of sphincter length.

This system may work in clinical practice, by picking up very short sphincters or those which are profoundly hypo- or hypertonic. However, in a study which may be addressing very small changes in the sphincter parameters, a more technical approach is required. This is where a motorised catheter puller is essential. These are computer controlled devices that withdraw the catheter from the nostril, at a pre-set velocity, between two points. Whilst the catheter moves, the software is able to constantly record the data and therefore build up a profile of data over the whole sphincter length. During this pull-through period the patient is requested to breath with a pattern of normal respiration, which causes oscillation of the pressure tracing. Analysis of the trace can pick-out either end of the sphincter, to the nearest millimetre, and also the location of the respiratory inversion point (RIP). The lower border of the sphincter is picked out as the breath in which the end expiratory pressure remains above that of gastric baseline pressure. The upper border of the sphincter is marked as

that breath in which the pressure drops below oesophageal baseline pressure in end expiration.

The LOS normally passes through the diaphragmatic hiatus, which exerts several effects on the pressure tracing. Firstly, the addition of pressure to the HPZ by a pinchcock effect. Secondly, it acts as a barrier between the two zones of intra-abdominal and intra-thoracic pressure. The RIP can be detected on the tracing, which correlates, with good accuracy, to the hiatus. This manifests itself on the pressure recording as the first breath cycle in which the pressure deflection decreases in expiration. This RIP mark is used to be able to analyse abdominal sphincter length. This calculation is not possible in the rapid motorised pull-throughs ($\geq 1\text{cm/sec}$), which are often performed with breath holding, and no reliable production of an RIP.

Manometry pull-throughs, in our studies, were performed using a motorised puller (Medtronic, Denmark) at a puller rate of 1mm/sec over a 10cm pull distance. There is a slightly slower response to pressure changes with the syringe driver set-up, which in itself produces a smoother trace. This slower response time makes this system unsuitable for acquiring data looking at both large and rapid changes in pressure such as those seen at the upper oesophageal sphincter during a swallow. However, at the LOS where the pressures are lower, in combination with a slow pull-through speed, which also allows for accommodation to changes in pressure, this system produces accurate recordings (Figure 2.6). There may be a very small underestimate of pressure at the LOS with this system, but it is accepted that this small error will be incurred for all analyses over the range of volunteers and patients studied. Despite this minor reservation, the values recorded for the maximum

end expiratory pressure of the LOS's of our range of healthy volunteers match those ranges established in clinical practice.

The nature of capillary tubing makes it very difficult for water to flow through it unless under high pressure, such as those created by a hydraulic water chamber (approximately 100kPa/15psi). These pressures are not attainable by the Braun syringe driver used in our design, restricting us to the narrow, one millimetre internal diameter Lectro-caths. Retrograde water flow through a pressure transducer fed by capillary tubing would only occur at pressure way above those found in the upper GI tract, because as well as countering the perfusion pressure of the pump, the resistance to flow of the water in the tubing, due to surface tension, also has to be overcome.

Idiosyncrasies of our design only make it suitable for use in this type of study, where the arrangement of the distal four recording channels on the manometry catheter are in the stated fashion. As the internal diameter of the supply tubing proximal to the pressure transducers in our design was one millimetre, there was a possibility that in some situations either retrograde flow, or more likely a failure to transmit pressure to the sensor, could take place. This was discovered in bench top testing. Despite the similarity in High-Low calibration and water column exposure, a 'single-channel occlusion test' gave differing results between the two systems. The Perfusor syringe driver was set at 99.9 mls/hr, which is equivalent to the sum flow rate of four channels when the hydraulic water chamber is at its standard pressure setting of 15psi. When the individual water flow from one of the four radial channels is completely occluded there is a response relayed to the PC, but not as much as would be expected from a standardly perfused transducer. For the reasons discussed before, when a single channel is occluded, with

our design, and there is no obstruction to flow at the other three channels, the flow around the loom is preferentially through these three and not against the resistance of the occluded site (Figure 2.7). This could lead to an inaccurate pressure recording.

The anatomy of the LOS is such that it is often subject to a degree of asymmetry with regards to external circular muscle bulk (30). Along with this can go a slight asymmetry of the pressure profile along the length of the HPZ, in differing directions. However, the location of the highest recordable pressure, (which with a slow motorised pull-through (SMPT) is the location of the maximum end expiratory pressure (MEEP), is relatively constant for each person's LOS. So, with all four of our data collection channels at the same depth during a manometry pull-through there is no risk of the type of pressure tracing loss, as initially feared. The only knock-on effect was that there may have been a partial equalization of pressure throughout the four channels during acquisition. This equilibrium may make the data tracings from each quadrant look rather similar compared to a standard set-up. However, we calculated our LOS pressure as an average of the four separate MEEPs recorded, and with no pressure losses from the HPZ during a pull-through, no noticeable effect would be seen on the result.

2.4.1 Positive control studies:

In order to properly test the recording capabilities of the manometry system, it was necessary to undertake a 'positive' control study. This meant that we needed to perform an *in vivo* study that would bring about a known measurable effect on the features of the LOS. Following a meal, gastric distension causes changes to the proximal stomach. Stretch placed on the muscular wall by the intragastric meal, as well as the effects of hormone release, cause the LOS to shorten. This is similar to the neck of a balloon as it is inflated. As well as shortening, there is also a reduction in the pressure of the LOS. The combination of these two events renders the subject more susceptible to reflux events. With regards to LOS shortening, in healthy volunteers, where the LOS spans the diaphragm, the shortening favours the LOS on the abdominal side.

2.4.2 Method:

We undertook baseline recordings of the LOS in 3 volunteers under fasting conditions. This was performed with the syringe pump perfusing 4 radial channels, which acquired the data, as previously described. A fasting, SMPT was undertaken in a reclined position, during shallow respiration, and with a pull speed of 1mm/sec. Distilled water was flowing through the manometry channels at a rate of 0.42 mls/minute. From this pull-through, data regarding the LOS could be assessed, including the total sphincter length, location of the RIP, length of the abdominal portion of the LOS and also the MEEP relative to the fasting IGP.

The catheter was then reinserted into the stomach, taped to the nose, and the perfusion stopped. The volunteers were sat upright and ate a meal of battered fish and chips, with a glass of still water. Five minutes after meal consumption the patients were reclined and perfusion of the catheter recommenced. The catheter was then withdrawn, using the SMPT technique as described before.

2.4.3 Results:

In all 3 volunteers there was a reduction in total sphincter length from a mean of 5.0cm (SD 0.13cm) to a mean of 3.9cm (SD 0.26cm) ($p < 0.006$). Abdominal sphincter length reduced from a mean of 2.73cm (SD 0.49cm) to a mean of 1.74cm (SD 0.86cm) ($p < 0.085$), and the MEEP reduced from a mean of 23.2mmHg (SD 6.27mmHg) to a mean of 11.2mmHg (SD 6.35mmHg) ($p < 0.056$) following the meal.

2.4.4 Conclusions:

The system design is capable of and sensitive enough to detect the physiological changes of the LOS which predispose to reflux. It is therefore suitable for our fasting study. The issue of NO delivery is discussed later in this chapter (2.6.1).

2.5 Meal study manometry design:

2.5.1 Measurement of transient lower oesophageal sphincter relaxations (TLOSRS):

2.5.1a Background:

The reflux of gastric contents into the distal oesophagus is a normal physiological phenomenon. Damage and compromise to the squamous epithelium is prevented by effective clearance of the refluxate due to peristalsis. The acidic pH is also neutralized following the delivery of swallowed saliva, and to a lesser degree by bicarbonate secreted from the oesophageal epithelium. There are three main methods by which reflux is said to occur. Firstly by sphincter failure or incompetence. This often occurs in sphincters with lower resting pressures, those of shorter overall sphincter length, or those with shorter 'abdominal' components to the sphincter (116). Failure of the sphincter leads to 'common cavity' events between the oesophageal and gastric compartments. This mechanism is more commonly associated with the reflux events in patients with symptomatic oesophagitis, hiatus hernias and Barrett's oesophagus. However, TLOSRS are the most frequent generators of reflux events in both GORD patients and the healthy population (122).

When swallowed, synchronized oesophageal peristalsis propels the bolus of solid matter, liquid or gas, towards the stomach. Sensory loops allow the LOS to anticipate the arrival of the bolus and relax to aid its passage. TLOSRS are relaxation events of the LOS not preceded by oesophageal peristalsis. They are more common in the post-prandial state and also with an erect posture. TLOSRS are likely to serve as a means of venting gas from

the stomach, which can be associated with acid reflux. If they give rise to a reflux event this can subsequently trigger peristalsis to aid acid clearance. A pharyngeal sensor to assess the co-ordination of swallowing allows an observer to identify these scenarios. The mechanism by which TLOSRS occur is not well understood. It is thought that the vagus nerve plays a major role in coordinating them (133). It would appear that afferent receptors react to mechanical forces in the proximal stomach to initiate TLOSRS events (120). Some feel that shortening, and loss of the abdominal portion of the sphincter, whether by gastric distension or hormonal means, is key (116;124). There is also evidence that TLOSRS last for longer than normal LOS relaxation events (213).

The manometry recording channels with our first system, designed to examine the LOS in the fasting state, were all located at the same level. For the measurement of TLOSRS a different catheter configuration is required. We were keen to deliver NO to the same area of mucosa as before, and record data concurrently, but with a slightly different setup. A Dent Sleeve catheter is often used to measure pressure across the LOS, recording the highest pressure along the sphincter at any one time. The pressure recording 'zone' for the LOS is comprised of a covered lumen and the water supplying this runs off into the proximal stomach, and has no direct contact with the LOS mucosa (Figure 2.8). There are often uncovered oesophageal ports on the catheter as well and also a gastric channel which can measure pressure in the standard way. With a covered unit sitting within the LOS, there is no opportunity for NO delivery as before. A catheter with serially spaced ports along its length can be used as an alternative.

We are intending to record TLOSRS after a meal. We have shown that following a meal, the HPZ shortens in length. This would mean that there may not be as many recording channels sitting in the LOS as in the fasting state. Using a catheter with the 4 distal channels spaced sequentially 1cm apart allows for slight axial movement of the catheter, with continual measurement of both intragastric and LOS pressures. Test solutions would be delivered through the lower 4 channels of the catheter (Figure 2.9), which is a different distribution to the previous protocol. Surprisingly, this does fit with the physiology of the post-prandial state. It would now be expected that our NO delivering ports would be both above and below the SCJ.

We previously mentioned that the NO chemistry is a pH-dependent reaction and that what is now suggested for the channel distribution goes against this. A study by Fletcher *et al* revealed that a 'pocket' of highly acidic unbuffered gastric juice existed in the proximal stomach following a battered fish meal. The limits of this showed that the proximal pH step-up point occurred on average 1.8cm proximal to the SCJ (23). This is clearly important with respect to both the exposure of the squamous mucosa to highly acidic and potentially damaging gastric juice, and also to the extent as to which parts of the gastro-oesophageal mucosae are exposed to the high levels of NO.

2.5.2 Method:

We selected a catheter made of the same polymer as the 2281-R model for its NO delivery properties. This was a 2282 model, single use, eight channel manometry catheter (Mediplus, UK). It has its most distal port at 5cm from the tip. Subsequent port spacings are 1cm, 1cm, 1cm, 2cm,

5cm, 5cm & 5cm proximal to this. At the lower end, this gives 5 ports within 5cm. We aimed to position the catheter with the most distal channel in the proximal stomach, and this would record gastric baseline pressure. The next 4 channels should sit within the LOS and record pressure locally. The remaining proximal channels would record oesophageal contractions and with a pharyngeal swallow sensor in place, both primary and secondary oesophageal peristalsis, as well as TLOSRS would be identifiable.

As discussed, there can be problems encountered with the 4-way splitting tubing arrangement in Figure 2.5. When any of these four channels are not under constant pressure there is a failure of the pressure built up in the 4 columns of water to be transferred back to the pressure transducers (Figure 2.7). As, in the second protocol, the catheter recording points would be spanning from gastric pressure, across the LOS and maybe into the distal oesophagus (all at differing pressures), this system had to be changed to record TLOSRS. So, each of the 4 channels was supplied individually by a separate syringe driver (Figure 2.10). The flow-rate through each channel was set to 99.9 mls/hr for each channel to increase the delivery of NO to the LOS and proximal stomach. This is an attempt to mimic increased salivary flow and nitrite delivery that occurs at the time of a meal. With each syringe containing only 50mls of solution, a change-over period would be needed half way through the 1 hour observation period, where each syringe is renewed, and a minor drop in NO delivery concentration might occur.

Concentration profiles of NO from the 2282 catheter tip can be seen in Figure 2.11. NO concentration from the catheter tip was assessed using the Greiss reaction (214). Samples were collected into phosphate buffer solution pH 7.4, with the catheter at 37°C in a water bath. A profile of NO delivery and

total exposure of NO can be calculated. These methods are described further in 2.6.1.

2.5.3 Results:

- The calculated concentration of NO at the tip of the 2282 catheter, for the meal study, is 1.1 millimoles/litre and reaches this level after several minutes of infusion
- The total exposure of NO for the 1 hour period is calculated as being 400 μ moles, (Average concentration of Nitrite / NO is 1 millimole/litre with 400mls of solution delivered). This is comparable to the 125 μ moles of NO predicted after a KNO_3 meal, which could be as high as 500 μ moles, due to the variability of nitrate reduction caused by the buccal bacteria.

2.5.4 Discussion:

This system has been validated as a manometry set-up and also as a suitable delivery system for a physiological NO solution. We have observed the slightly slower pick-up of pressure with the syringe driver system, and these limitations have been discussed. However, this system is very good at detecting the drop of pressure being recorded as in a TLOS, or any other sphincter relaxing episode (Figure 2.12). With pH probes attached to the manometry catheter, episodes of acid reflux could also be analysed in relation to the swallowing, peristalsis and sphincter relaxation events. Our delivery of NO is as a smooth curve, whereas in reality it is more likely to be bursts of NO exposure, as the boluses of nitrite rich saliva enter the acid

environment of the stomach. This is merely hypothesis, and whether one way is more likely to have an effect than the other, is open for debate.

2.6.1 Nitric oxide delivery – solution to be used for fasting study:

Proof of biological effect -

In our laboratory, we have formulated a unique solution that will generate soluble nitric oxide *in vitro*. The constituents of this are described below. This solution mimics the luminal chemistry occurring within the lumen of the upper GI tract. There is no evidence within the literature that a solution of this type has been used in such experiments before. We have previously squirted this NO solution on to gastric rugae during routine endoscopy to see if there were any obvious relaxation effects. This proved difficult to quantify due to the constant slow remodelling of the stomach's inner surface. We, therefore, took this solution and placed it on the skin of the forearm of a volunteer, with three other controlled blebs. One of hydrochloric acid, one of the NO generating solution minus the nitrite and one of acidified nitrite plus thiocyanate, but without ascorbic acid. After less than 30 seconds a change could be seen and after two minutes the difference was striking. A well-defined red area, limited to the area of NO exposure, can be seen which presumably is due to the dilatation of pre-capillary arterioles (Figure 2.13). This is thought to be due to the smooth muscle relaxation properties of NO and serves as proof of a biological effect. This fuelled our theories that NO generated within the lumen of the upper GI tract may be able to cross the epithelium and have some effect on the underlying tissues.

Nitric oxide is a small lipid soluble molecule able to traverse lipid membranes *in vivo*. Although not able to permeate glass it can pass through

certain plastic and rubber tubings to varying degrees. In our experiments, we needed to be sure that the system that had been designed and tested to measure pressure, would also be able to deliver NO to the end of the manometry catheter in concentrations suitable for our study. As described, our delivery system contained several components made from various plastics. The total distance from syringe driver to catheter tip measured approximately 2 metres. With a low flow-rate the time delay for the solution travelling from syringe to catheter tip would also allow any escape of NO to be amplified.

NO levels were initially measured using a pre-calibrated bench-top probe (ISO-NOP, World Precision Instruments, USA) (215). Samples were collected from time zero, and every 5 minutes upto 25 minutes, and then a final sample taken at 28 minutes. For comparison, samples were analysed from the syringe at zero and 30 minutes to compare and assess and deficit over the length of the tubing. As described earlier, the contents of the syringe were sodium nitrite 1.6mM, thiocyanate 1mM, ascorbic acid 5mM, hydrochloric acid pH of 1.6 and EDTA 1mM. This solution should generate 1.6mM NO.

Certainly, when trying to attain higher concentrations of NO by increasing the nitrite concentration (e.g. 4mM), and with more ascorbic acid present to ensure the reaction is driven, the inside of the plastic syringe was completely covered with small bubbles, which over the time of the 30 minute infusion had coalesced to form large bubbles throughout the whole loom, including the pressure transducers. This made the system unworkable, and it was assumed that what was being witnessed was bubbles of NO coming out

of solution. So, at room temperature and atmospheric pressure, the saturation point for NO in distilled water was accepted as 1.6mM.

2.6.2 Method:

A standard manometry catheter from clinical practice was used to deliver the NO solution (2281-R, Mediplus, UK). These catheters are made from medical grade PVC. In order to mimic physiological conditions, the NO probe was calibrated at 37°C and the distal 45cm of the catheter, which would be in the patient, were also placed in a water bath at 37°C. This was also done with the catheter at room-temperature just to make sure that an increase in temperature did not cause a significant loss of NO from the system.

Cations in solution can theoretically interfere with NO generation and so EDTA was included as a chelator to assess its benefits on NO generation. In order to try and reduce all types of NO losses from the system we removed the dissolved oxygen from distilled water. This is because the oxygen in solution reacts with the NO to form nitrogen dioxide and this is then recycled back to nitrite. In this final stage there is a 50% loss of nitrite to form nitrate, which is then lost from the ongoing recycling process, and this reduces the total nitrite available to produce NO (208). In preparing the syringe contents, one litre of distilled water was gassed with zero grade Argon (BOC Gases, UK) through a gassing 'stone' for 10 minutes to accomplish this. This was initially verified by a calibrated oxygen probe (ISO₂, World Precision Instruments, USA) in the laboratory and used thereafter as the standard gassing time. This 'degassed' water was used to make up the

syringe solutions, prime the tubing lumen and also to fill the manometry reservoir. The hydrochloric acid was gassed individually.

In another assessment, distilled water was pumped through the 4 proximal 'oesophageal' ports on the manometry catheter. This was done to investigate two questions. Firstly, did the concentration of NO drop when there was water in these other 4 channels and secondly, if so, was there any significant quantities of NO in the out-flow from these other channels? We know that NO can pass through certain plastics and we considered that this may happen within the catheter length as each of the eight individual lumens sit in close proximity to each other (Figure 2.14).

Losses of NO gas to the atmosphere were postulated as a cause for the lower levels of NO. Therefore, a different sampling method was employed. The concentrations of NO at the catheter tip were reassessed using the Greiss reaction (214). Sample collection without pipetting reduces losses of NO to the atmosphere. Therefore, samples from the catheter tip were collected into glass scintillation vials containing known volumes of phosphate buffer solution at pH 7.4. The submerged catheter tip was agitated to mix the solutions and the amount of NO generating solution added over a 30 second period. To allow for inaccuracies of the syringe driver flow rate, the volume of NO solution added was calculated by a 3 decimal place weighing balance. Calculating the change in mass, and knowing the density of the NO generating solution, the volume deposited could be calculated. By raising the pH of the samples, all NO is captured and stabilized by conversion to nitrite. Spot nitrite analysis on each sample was then carried out by the Greiss reaction using spectrophotometry (214).

2.6.3 Results:

- Loss of NO due to dissolved oxygen was apparent even at low concentrations of NO formation – approximately 50%, 6 v 12 μ moles/litre (Figure 2.15a).
- The addition of EDTA, which removes any small metal cation impurities from our salts does increase the NO levels (from 12 to 16 μ moles/litre) and this is independent of temperature – increase by upto 33% (Figure 2.15b).
- With the bench-top NO probe it can be seen that it takes approximately 6-8 minutes for there to be recordable levels of NO at the catheter tip, which gradually rises and plateaus at 20-25 minutes, with a NO concentration of 350 μ moles/litre, before slowly decreasing (Figure 2.15c).
- There is no alteration to the NO concentration associated with a change of temperature (Figure 2.15c).
- Analysing the effect of water within the 'oesophageal' perfusion channels showed that there was no drop-off in the NO levels and that there was no recordable NO in the out-flow from the other channels (Figure 2.15c).
- Analysis of nitrite concentrations by the Greiss reaction showed that the NO concentration at the catheter tip plateaued after 15 minutes of the infusion, at a concentration of 800 μ moles/litre (Figure 2.16a). Also, the concentration of NO in the syringe at the start of the infusion was 1.4 millimoles/litre and 1.2 millimoles/litre after the 30 minute infusion (Figure 2.16b).

2.6.4 Discussion:

The absence of NO detection within the 'oesophageal' ports solution was very useful to know, as we did not wish there to be any inadvertent NO exposure to the oesophagus. However, if it were to be present there by the reflux of the infusion during the experiment then that would need to be taken into account when interpreting the data.

It was accepted that there were errors involved in the sampling procedure using the bench-top NO probe. Immeasurable, but consistent losses of NO to the atmosphere during pipetting occurred. Even when a sample was taken from a well-mixed, freshly made syringe, there was a large discrepancy compared to the expected concentration of NO. Certainly, a lot less than the expected 1.6mM of NO with 100% conversion of nitrite, was found. These lower than predicted values of NO were also found in the solution pumped through to the catheter tip, and were in fact lower again than those found in the syringe. There was a degree of dilution of NO as it passed through the loom and mixed with the deoxygenated distilled water in the loom and pressure transducers, which measures several millilitres. This would account for only a small degree in the drop of the NO level from the syringe to that maximally attained at the catheter tip. It was thought that this further drop in measured NO was due to losses through the plastic tubing by diffusion.

At low, micromolar concentrations of nitrite, the conversion to NO, under the correct conditions described, is virtually 100%. The assessment of NO generation at the higher levels (millimolar) that we are dealing with has not been looked at. We can theoretically extrapolate the reaction to assume 100% conversion at concentrations above 1 millimole per litre. We mentioned

that we would expect there to be an underestimate of the NO concentration by our first sampling method. We also expect to see higher concentrations of NO, in the form of nitrite, in our samples assessed by the Greiss reaction. Due to a lack of complete certainty with these second results, it is probably safe to assume that the actual NO concentration is somewhere in between the two.

Seeing the levels of NO being generated we had to decide if these were appropriate for our protocol. The majority of the NO was present between 8 and 28 minutes of the infusion. With an average concentration of 700 $\mu\text{mol/litre}$ in 33.3 mls of solution delivered, this would give a NO load of 23 μmoles . You can see how this compares to a predicted 24 hour exposure of NO derived from salivary nitrate with both a basal nitrite secretion and after a 2 millimole nitrate meal (Figure 2.17).

Of a 2 millimole potassium nitrate meal we would expect, on average, approximately $1/16^{\text{th}}$ to end up as luminal NO ie: 125 micromoles. This appears in the lumen about 30 minutes after ingestion and dissipates over 3 to 4 hours. A 4 hour period contains 12 x 20 minute segments, which would average at 10 micromoles per segment, comparable to our exposure. Useful to note is that in the fasting state less ascorbic acid is secreted from the stomach and so less NO would be produced (216). Our initial look at the LOS is in the fasting state and so we are likely to be equating, or slightly exceeding, normal NO exposure. The effects of NO on LOS pressure are difficult to assess in the post-prandial state due to TLOSRS. Manometry pull-throughs can be performed lying down, but this is less physiological, as people often have an erect posture immediately after a meal. There certainly

is a place for investigating aggravating factors of supine reflux, but we were not focusing on this with our protocols.

It would be expected that the effect of NO is dependent on total dose delivered, flow rate and concentration of the delivered solution. In the fasted state, as in the first study set up, the flow rate is relatively slow and comparable to a standard water-perfused manometry system. Also, the NO delivering solution is in direct contact with the mucosa and its overlying mucous layer. Therefore, it should be able to have its maximum effect, having the opportunity to diffuse across the epithelial tissues, although we do not know the optimum NO concentration / diffusion gradient to cross the epithelium, and this would be difficult to measure. As the active solution would be delivered as the perfusate through the manometry channels, it would be given as a constant infusion. This varies to normal physiology, where swallowed nitrite in saliva would be delivered to the stomach in more discrete boluses. This was an accepted limitation of the methodology.

At higher flow rates, the solution would simply run off into the empty stomach. There is a question as to what may happen when there is a meal present within the stomach. With the knowledge of the 'acid pocket' and knowing that there is a small potential 'space' in the proximal stomach, it is postulated that the NO that flows in, either by syringe or generated by salivary nitrite after a meal, may collect in this region over time and be able to exert its effects accordingly.

2.7 Summary:

The pathophysiology of GORD is not fully understood, but it is known that both anatomical and dietary factors play a role to varying degrees. In the presence of a healthy acid secreting stomach, dietary nitrates generate NO in the lumen of the upper GI tract and the concentration is maximal at the gastric cardia. The effect of this NO on LOS function is not clear and so we have designed systems to analyse this further. Oral supplementation with nitrates has been used before to address this question, but salivary nitrite levels are often unpredictable.

We believe that it is first necessary to assess the NO effect on a fasting LOS before embarking on the more challenging situation following a meal. The tubing looms described here give reproducible and reliable delivery of a NO generating solution. The pressure recording capabilities of the system vary only slightly to those of the low compliance capillary tubing arrangement in standard clinical use. It is a system that calibrates equally as well and gives good quality tracings suitable for clinical interpretation. These features combined, allow for the investigation of our test solutions' effects.

Table 2.1: Constituents of the NO generating solution in their final concentration.

Constituent	Final concentration
Ascorbic acid	5mM
EDTA	1mM
Nitrite	1.6mM
Hydrochloric acid	pH 1.8
Thiocyanate	1mM

Figure 2.1: Diagram to show the entero-salivary re-circulation of dietary nitrate (NO_3^-), its conversion to salivary nitrite (NO_2^-) and delivery to the stomach. Approximately 25% of dietary nitrate is concentrated into the salivary glands and a further 25% is then converted to nitrite. This conversion is variable, but on average, $1/16^{\text{th}}$ of ingested nitrate will end up as oral nitrite.

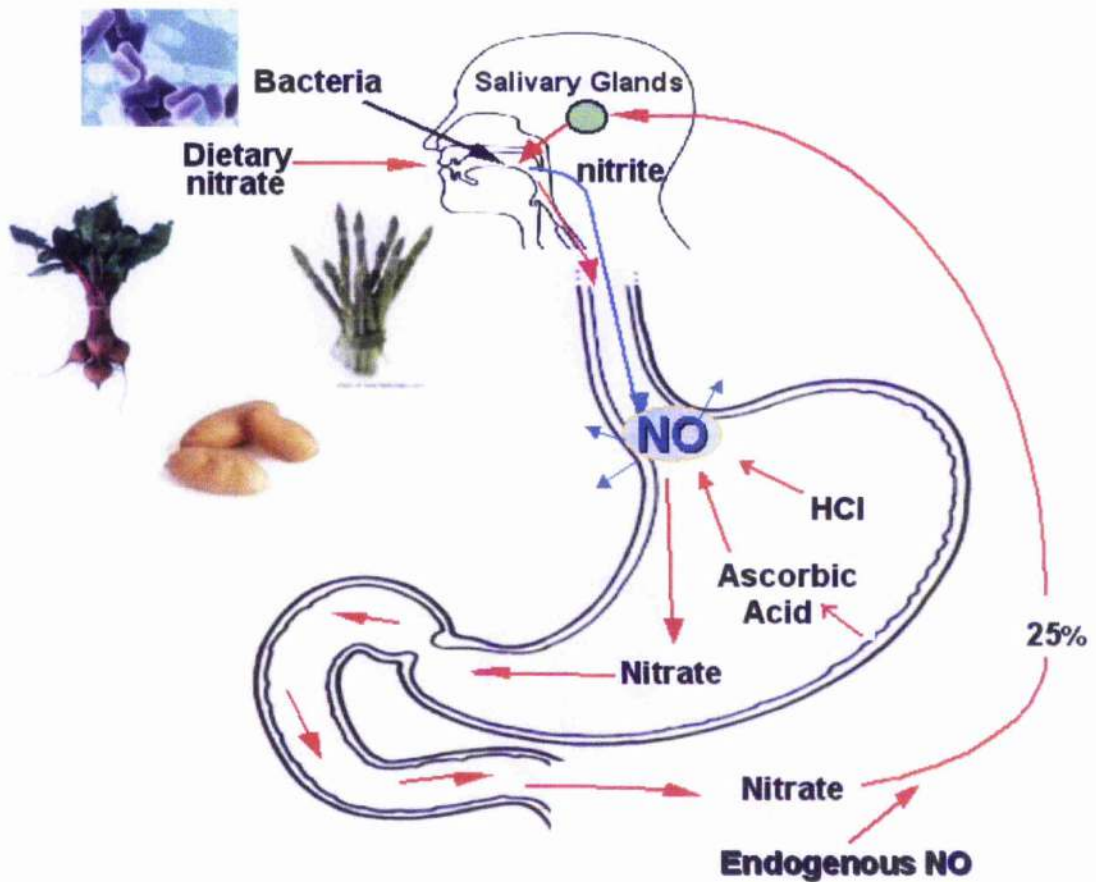
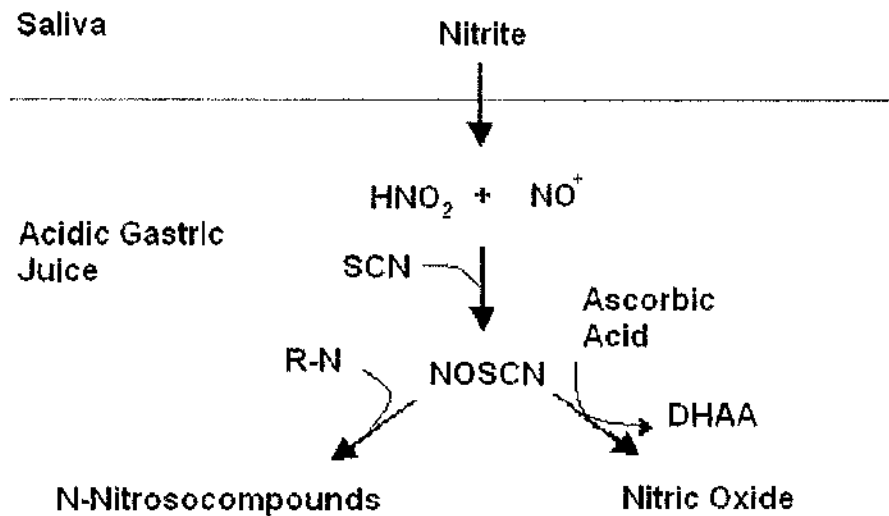


Figure 2.2: Demonstration of the nitrite chemistry that occurs in the acid environment of the stomach – Acid catalysed nitrosation.



In acidic gastric juice nitrosation maximal:

- at pH2.5
- in presence of nitrite
- in presence of Thiocyanate (SCN) in saliva
- in absence of Ascorbic Acid

Figure 2.3: Location of the four radial manometry channels in the LOS.

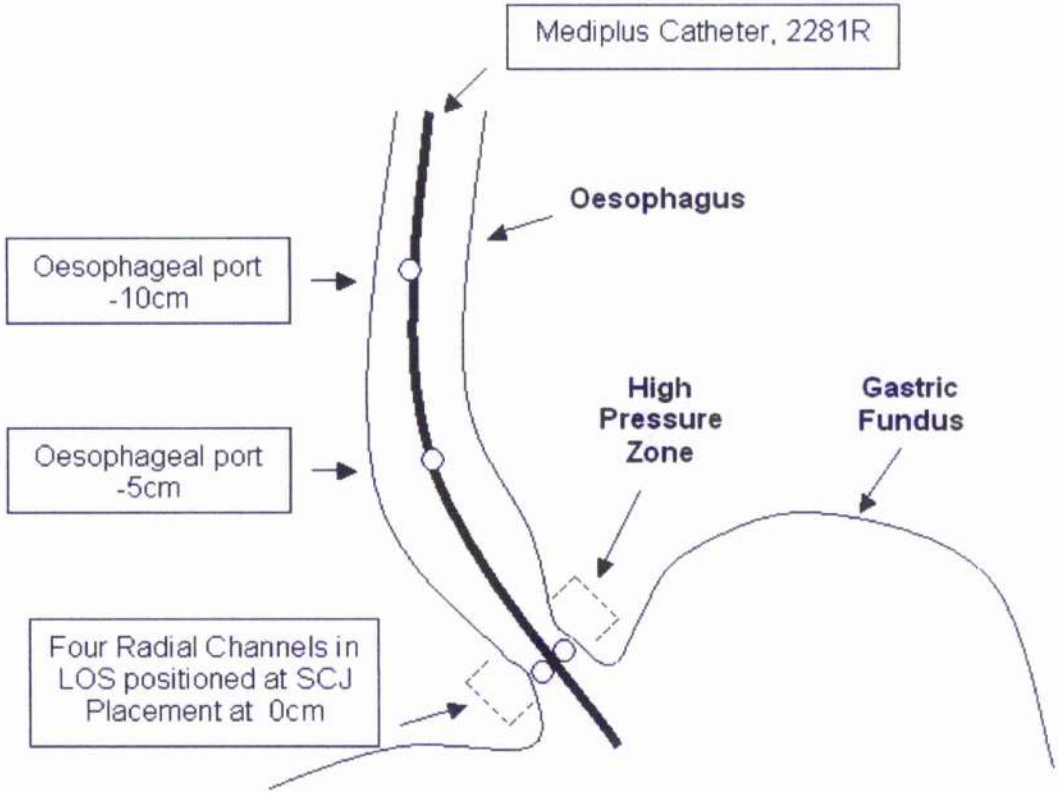


Figure 2.4: Diagram to show how the 'Traffic lights' system causes preferential flow through some fluid channels, with subsequent inaccurate pressure recording. Fluid flows in at port 1, with the majority of this solution exiting via channel 2. Decreasing amounts exit via channels 3 to 5.

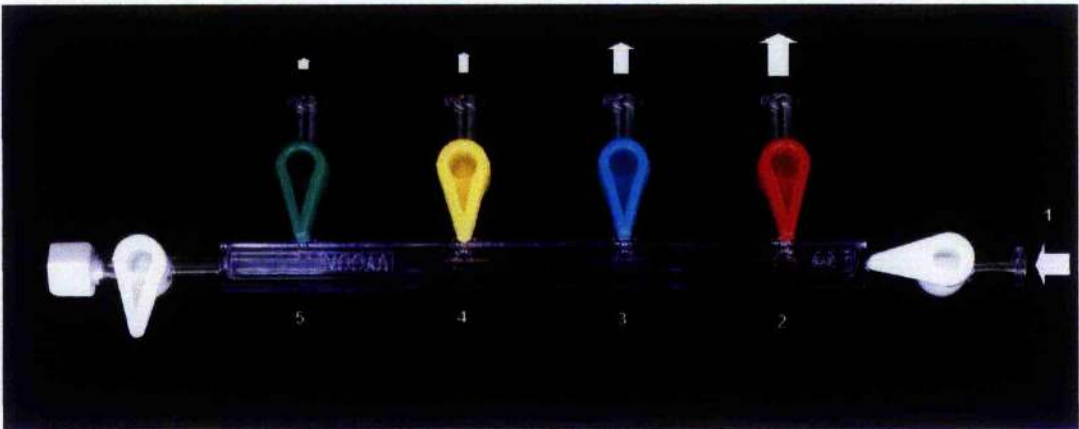


Figure 2.5: Arrangement of the tubing delivery system to the 4 LOS transducers with the Mediplus 2281R manometry catheter.

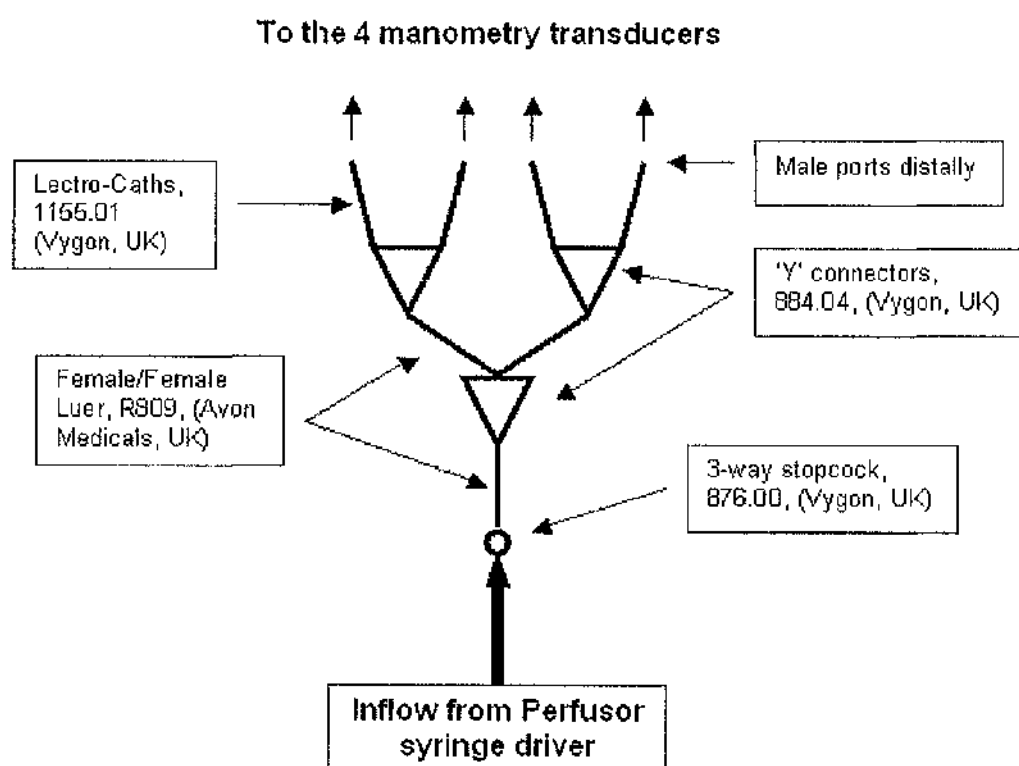
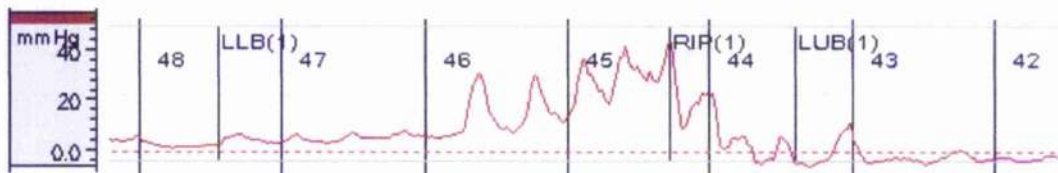


Figure 2.6: Tracings with manometry pump and syringe driver for comparison.

Single channel trace taken from the same patient on different days. Scale in mmHg. One centimetre pull-through markers on trace. LLB = Lower oesophageal sphincter Lower Border. RIP = Respiratory Inversion Point. LUB = Lower oesophageal sphincter Upper Border. Horizontal hatched line represents gastric baseline pressure.

Slow motorised pull-through of LOS with standard manometry chamber.



Slow motorised pull-through of LOS with syringe driver supplying radial channels.

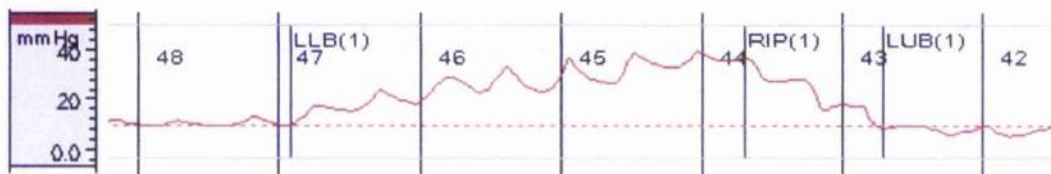


Figure 2.7: Diagram to show how the 4-way split loom can cause preferential flow up one side of the loom (A) and poor pressure recording on the other side (B), as well as poor NO delivery to this site.

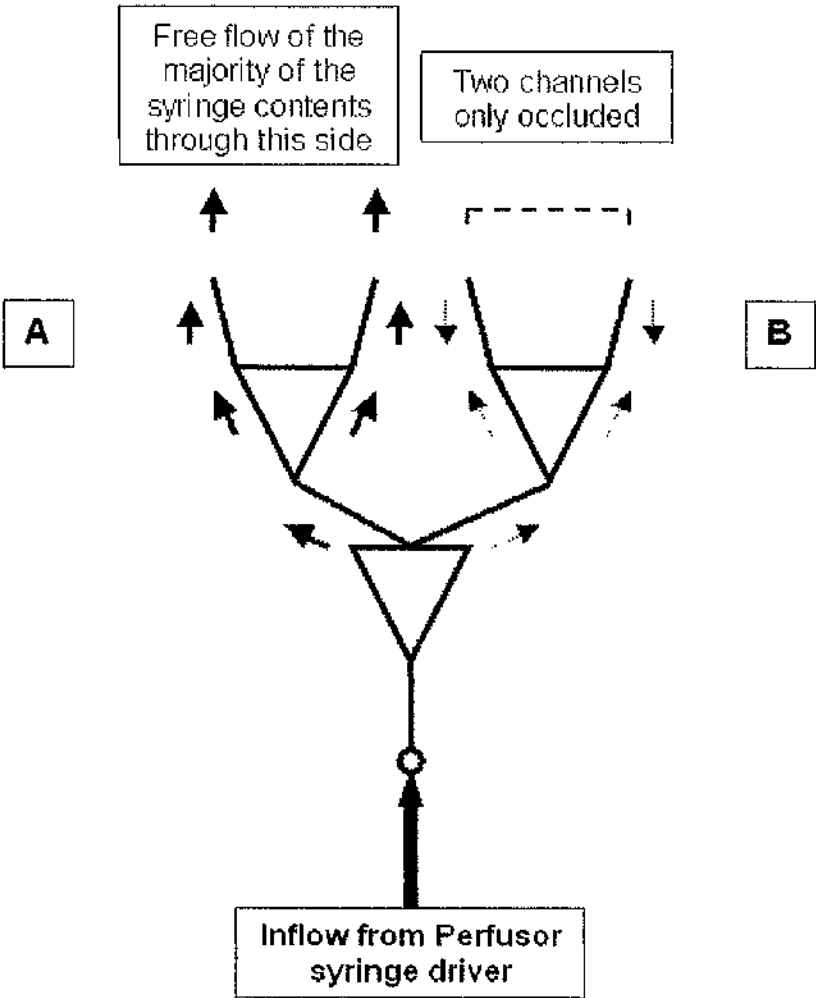


Figure 2.8: The use of a Dent sleeve catheter could measure LOS pressure and TLOSRs. However, NO in the solution delivered via the sleeves port would not contact the cardiac mucosa.

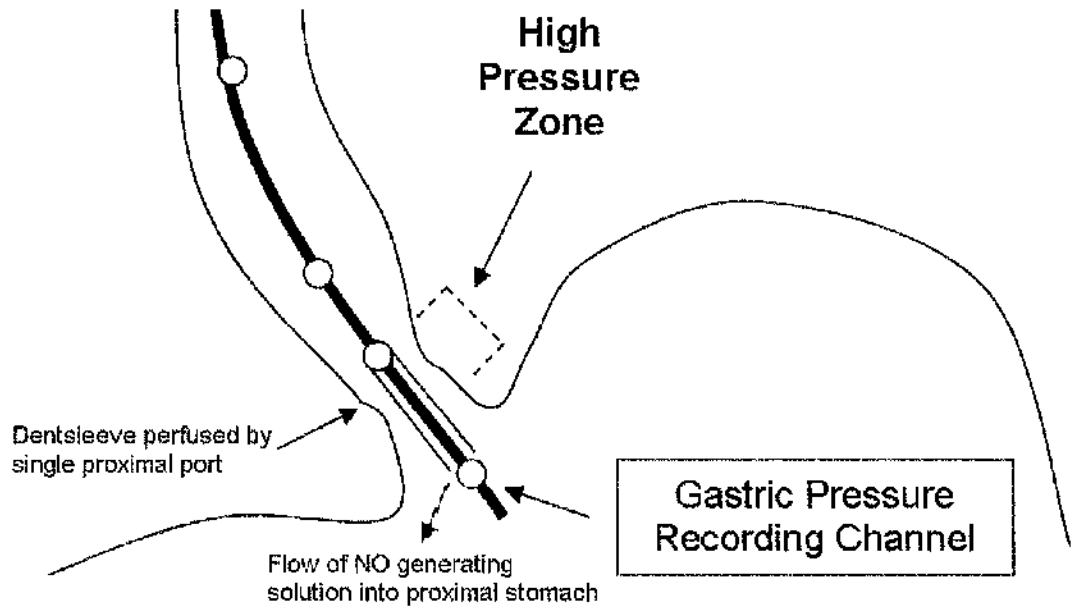


Figure 2.9: Location of the Manometry Channels for delivering NO and recording TLOSrs.

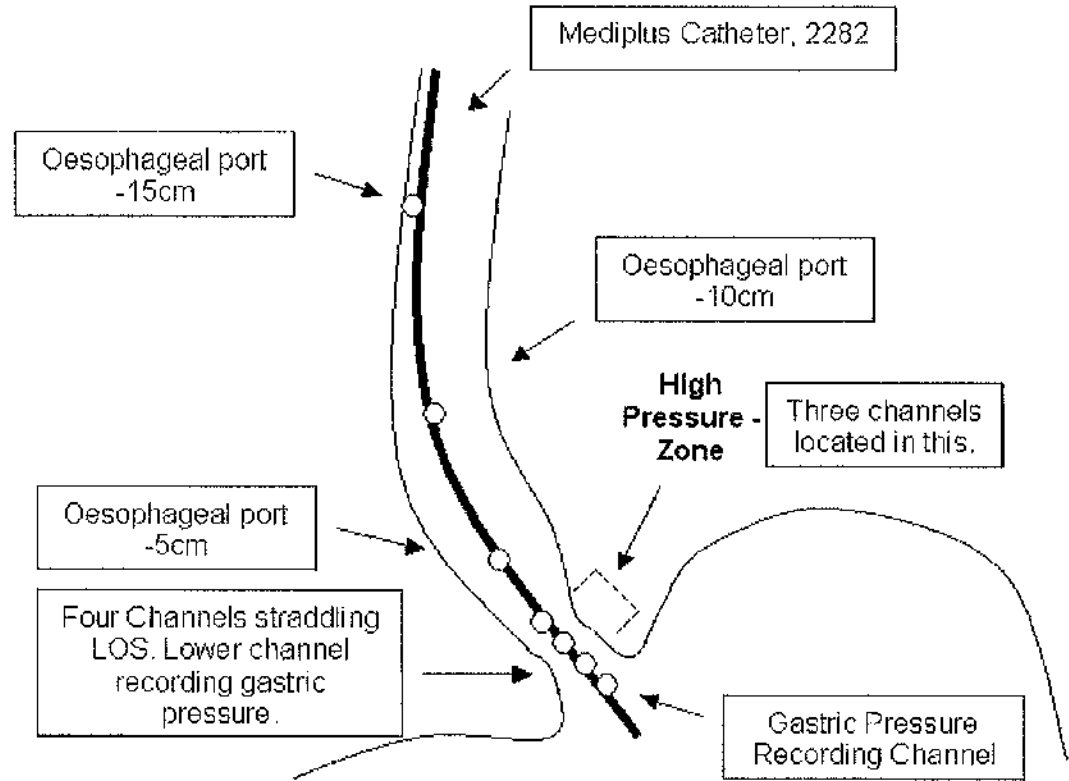


Figure 2.10: Arrangement of the NO delivery system to the 4 pressure transducers with the Mediplus 2282 manometry catheter.

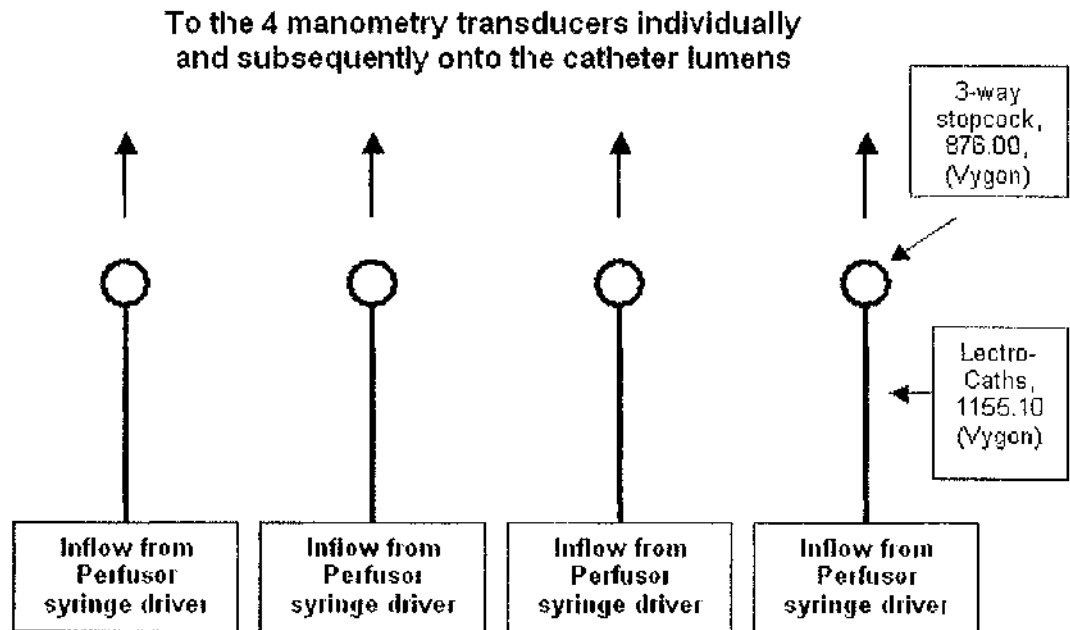


Figure 2.11: Graph to show concentrations of nitrite (assuming correlation with NO concentration) measured by capturing the catheter's solution (2282, Mediplus, UK) under phosphate buffer solution (pH 7.4) and then analysed by the Greiss reaction.

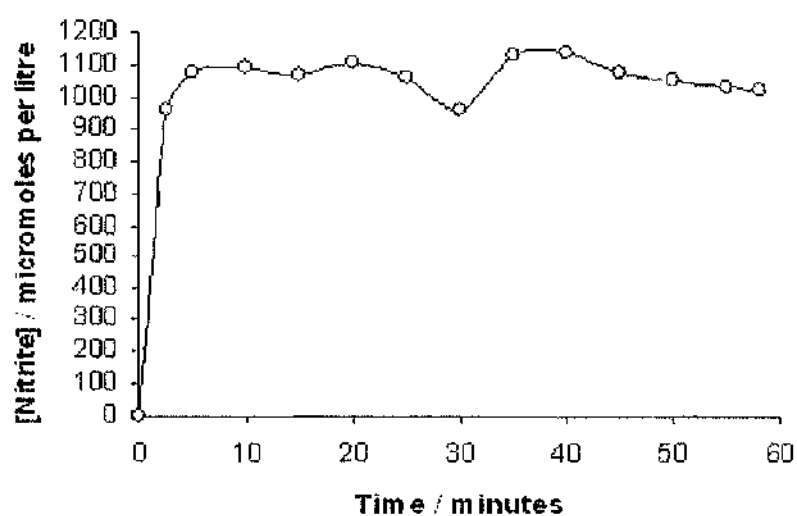


Figure 2.12: Example of TLOSRS recorded on Polygram Net using the 4 syringe arrangement. Taken from a tracing following a battered fish meal. Note varying scale for pressure for different channels. Channel positions are in centimetres from the nostril. Channel at 42cm positioned at the proximal border of the LOS giving an unreliable trace. Note LOS shortened from 4.1cm pre-meal to 2.1cm post-meal. Reflux episode apparent after TLOSRS.

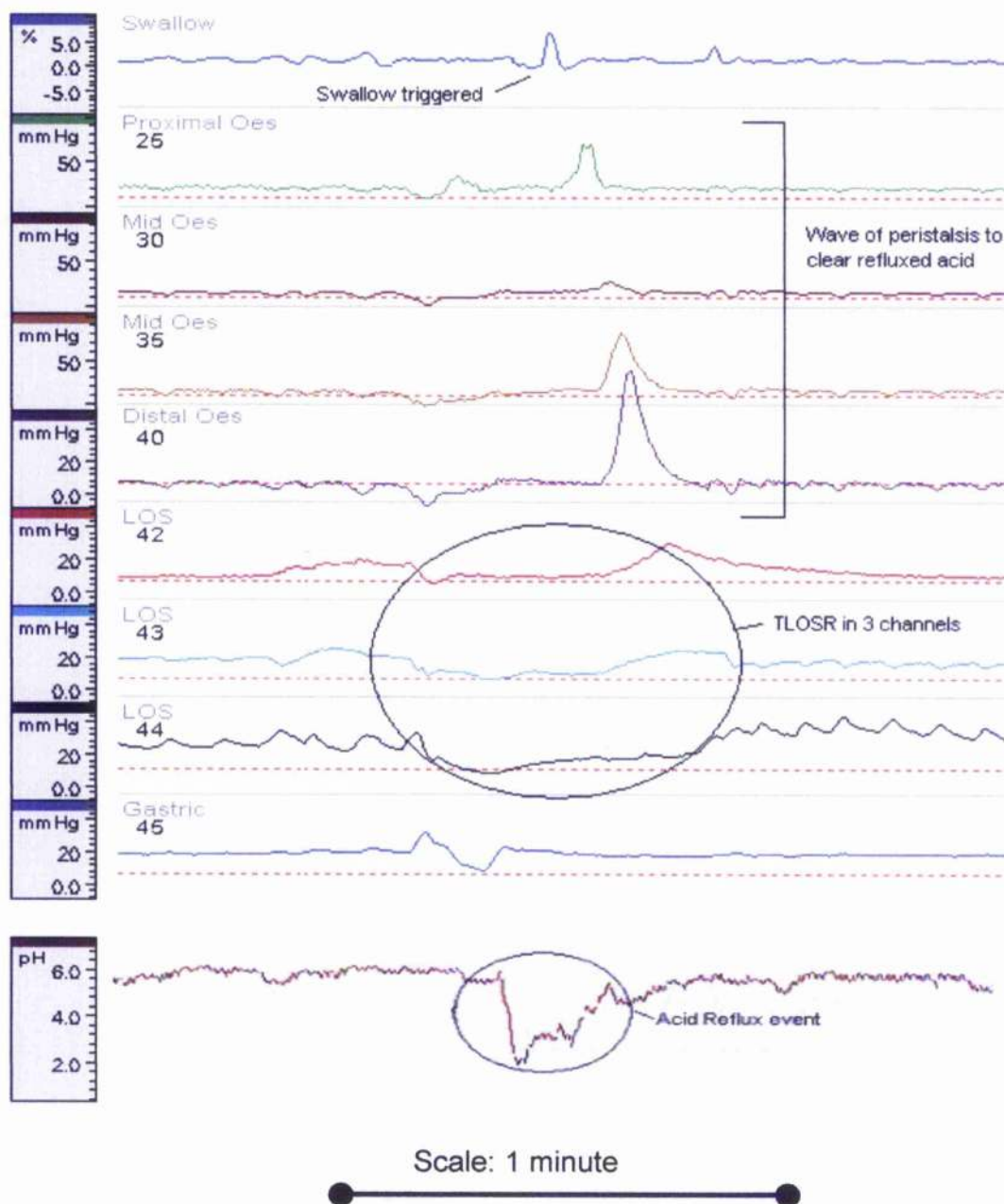


Figure 2.13: Example of the effect of NO generating solution on the skin of the forearm. Separate controls used for comparison.
Key: NO_2^- - Nitrite, SCN - Thiocyanate, HCl - Hydrochloric Acid, AA - Ascorbic Acid.

Solution constituents:

- 1: NO_2^- (1.6mM)
- 2: SCN (1mM), HCl (pH 1.6), AA (5mM)
- 3: SCN (1mM), HCl (pH 1.6), NO_2^- (1.6mM)
- 4: SCN (1mM), HCl (pH 1.6), NO_2^- (1.6mM), AA (5mM)



Skin pre-application

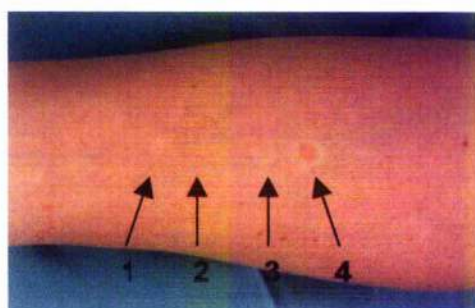
1 2 3 4



Solution application.
Time = zero



Solution application.
Time = 5 minutes



Solution removed.
Time = 10 minutes

Figure 2.14: Cross-section view of an 8 lumen manometry catheter. Water filled channels (W) run in close proximity to the test solution filled channels (S), and NO could potentially travel from one to the other depending on the properties of the lumen wall. The open-ended, empty central catheter lumen is also depicted (C).

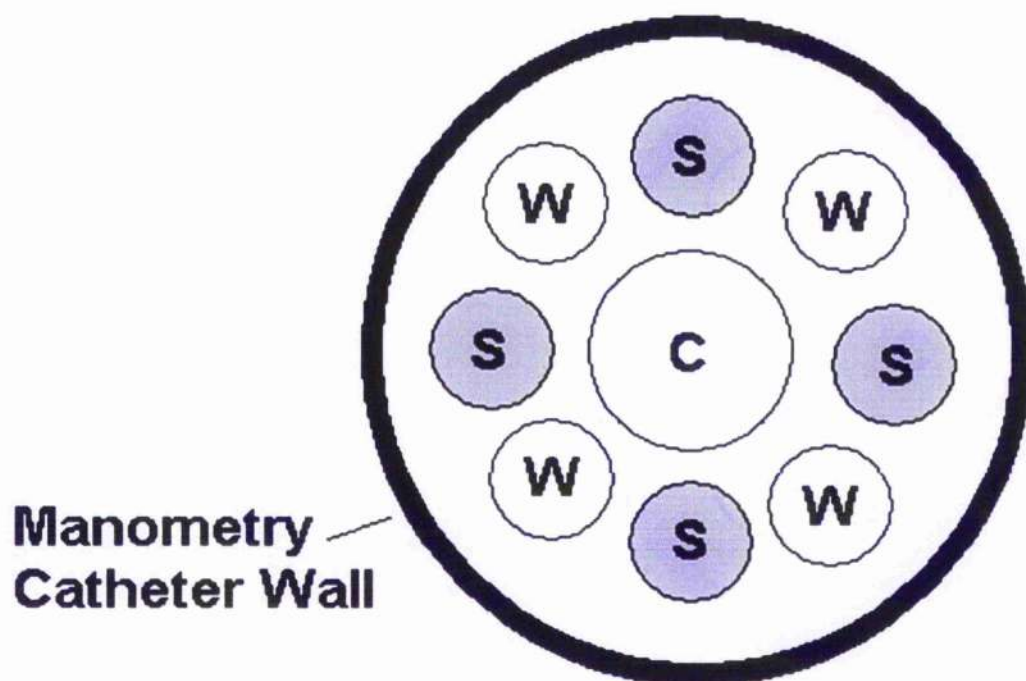
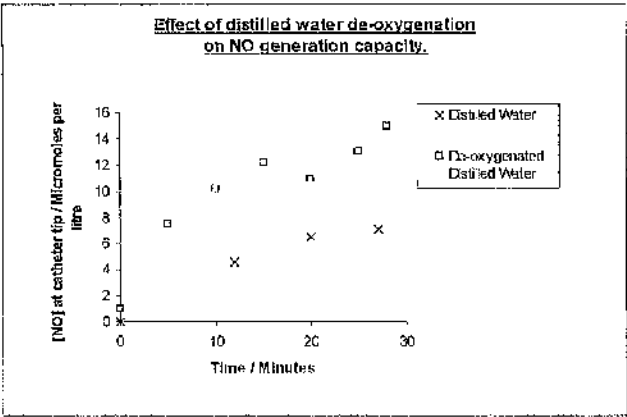
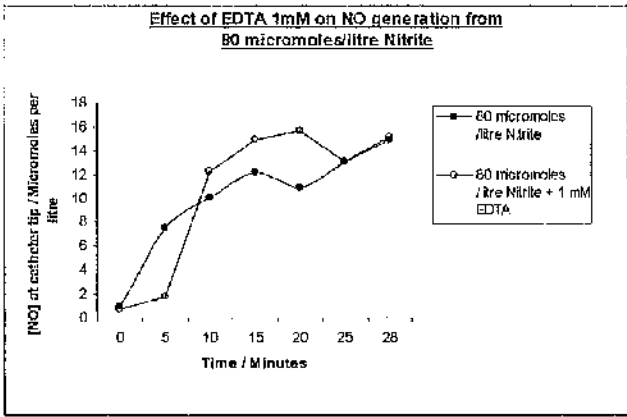


Figure 2.15: NO concentrations achieved at manometry catheter tip against time, under various intra-syringe conditions. See legend prior to each chart for details.

A: Common solution components for both series are Hydrochloric acid pH 1.6, Thiocyanate 1mM, Ascorbic Acid 5mM and Nitrite 80µM.



B: Common solution components for both series are Hydrochloric acid pH 1.6, Thiocyanate 1mM, Ascorbic Acid 5mM and Nitrite 80µM all made in de-oxygenated distilled water.



C: Common solution components for all series are Hydrochloric Acid pH 1.6, Thiocyanate 1mM, Ascorbic Acid 5mM, EDTA 1mM and Nitrite 1.6mM all made in de-oxygenated distilled water.

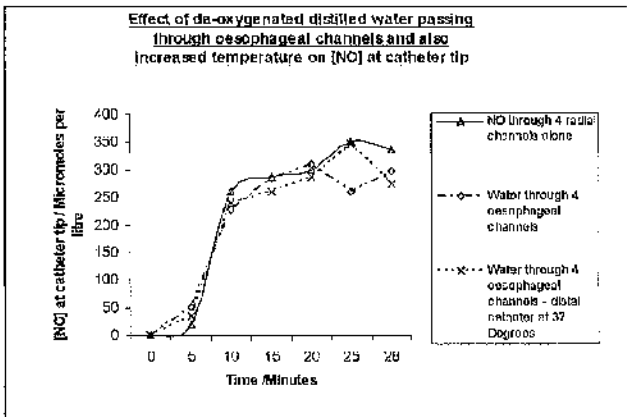
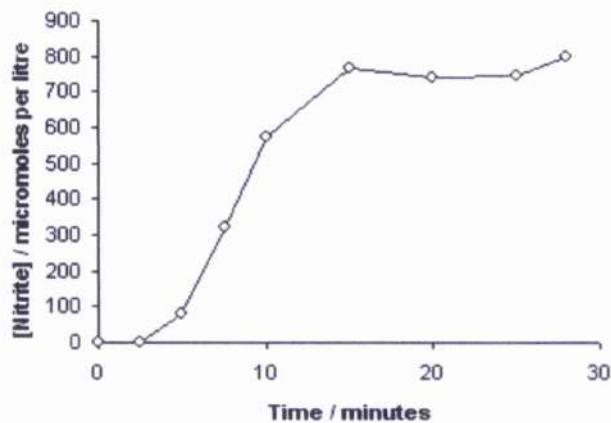


Figure 2.16:

A: Graph to show concentrations of NO converted to nitrite measured by capturing the catheter's solution (2281-R, Mediplus) under phosphate buffer solution (pH 7.4) and then analysed by the Greiss reaction.



B: Chart to show syringe driver concentration of NO at the start and end of the experiment, measured by Greiss reaction.

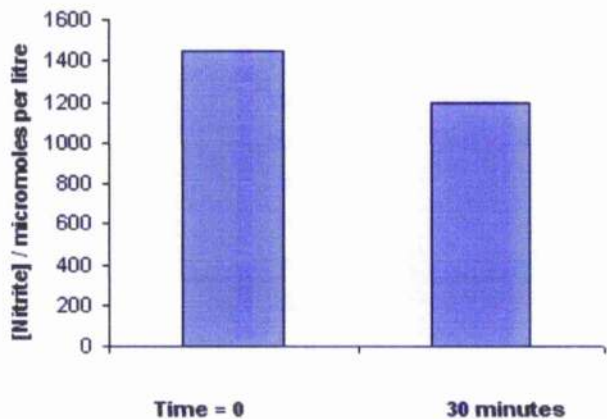


Figure 2.17: NO calculation for saliva etc and 24 hours.

NO concentrations in the Upper GI Tract

Salivary flow rate of 1-2 litres/day

= Average 1.5 litres/24 hours = 1500mls/1440 mins

Saliva Nitrite levels:

Basal = 50-500 μ moles/litre

After 2 millimoles KNO_3 meal = 200 μ moles/litre–2 millimoles/litre

Potential NO levels:

Basal = 0.05–0.5 μ moles of NO/min

Post KNO_3 meal = 0.2–2 μ moles of NO/min

Potential NO generation:

Daily low level 0.05 μ moles of NO/min x 1440 mins/day

= 72 μ moles/day

4-way syringe driver runs at 100 mls/hour = 1.667 mls/min

Exposure at end of catheter for 20 minutes = 33.33 mls

Concentration of solution at the tip of the catheter is 300-700 micromoles / litre

= 300-700 μ moles / 1000 mls

= 0.3-0.7 μ moles / ml

Total exposure to NO during the experiment

= 0.3-0.7 μ moles / ml x 33.33 mls

= 10-23 μ moles NO delivered during the fasting study protocol.

Standard Nitrate meal of 2 millimoles KNO_3

Expected to recycle 25% to salivary glands

Average conversion of 25% to nitrite in oral cavity

1/16th of meal ends up as nitrite over 3-4 hours

Potential conversion to NO (Amount of Nitrite)

= 125 μ moles

**Indirect assessment of NO exposure to the
oesophageal lumen by nitrite analysis
in a segmented silastic tube.**

Chapter 3

Chapter aims:

Within this chapter, is a series of experiments using segmented silastic tubes in the upper gastrointestinal tract. Our research group has previously used these tubes as an epithelial model which responds to the NO and nitrite chemistry in a physiological fashion. These would be in passed at the same time as our manometry catheters and experimental solutions run as per our planned protocols. Each of these silastic tubes contains phosphate buffer solution, which traps NO passing into each segment by converting it to nitrite. On removal of the tubes, each segment can be analysed for nitrite, which will act as a surrogate marker of NO. The distribution of NO in the tube will allow us to interpret with more confidence where our solutions are being delivered in both fasting and fed stomachs. This will allow us to interpret our physiological findings with more confidence.

3.1 Abstract

3.1.1 Background:

Iijima *et al* demonstrated that 20-30 minutes following a nitrate meal, in a healthy volunteer, luminal NO is detectable in the upper GI tract using a NO probe (162). This is most prominent at the gastric cardia, correlating with the gastro-oesophageal pH step-up point. Suzuki *et al* then took this one step further. They showed, that in a refluxing patient with Barrett's oesophagus, NO production can be detected with the same intraluminal NO probes, up into the oesophageal lumen (166). The Barretts' studies were performed with the patient fasted and in a recumbent right lateral position.

3.1.2 Aim

This series of experiments was designed to analyse the NO distribution profile, in the upper GI tract, produced by our experimental catheters, without intraluminal NO probes.

3.1.3 Methods

Our studies here involved only healthy subjects. Entry criteria required our subjects to be free of symptomatic acid reflux. Although the total HPZ length varied between subjects, none were found to have a hiatus hernia on manometry. Although the calculated doses of NO delivered were within physiological realms, the manner in which it occurred was not. Nitrite is converted to NO in the presence of acid and ascorbic acid. Nitrite is swallowed in saliva and therefore delivered down the oesophagus in boluses. Our experimental NO would be delivered as a constant infusion to the pH step-up point, in the fasted state, and in the predicted region of the 'acid

pocket' post-prandially. Also, saliva has a variable and different viscosity to that of our distilled water solution.

We were unsure if this would mean that the solution would simply run off down the lesser curve of the stomach and have no local effect whatsoever. Therefore, if there was no apparent effect from the test solution, we could not be sure whether it was because it was not being given a fair chance to exert any local effect. We designed some experiments to try and describe the distribution of the NO delivery and at least rule out a false negative result. This would involve a segmented silastic tube, being attached to the manometry catheters and our test solutions infused. Analysis of the tube segments was performed on withdrawal.

3.1.4 Results

In the fasting study, virtually no NO was detected within the oesophagus and the majority at the distal LOS. In the post-meal experiments it was shown that NO was found in the area of the expected acid-pocket. It was also present in the body of the stomach, but there was also evidence of NO reflux into the oesophagus. Concentrations were similar to those expected by oral ingestion of nitrate.

3.1.5 Conclusions

Our series of experiments have shown that the NO distributions and concentrations produced by our experimental systems are similar to those expected under normal physiological conditions. This reinforces the use of our experimental system to collect this data.

3.2: Assessment of NO delivery profile to the gastro-oesophageal junction, from a 2281-R manometry catheter, in the fasting state.

3.2.1 Aim:

To assess the distribution of luminal NO exposure around the GOJ following delivery of a NO rich solution, by a 2281-R manometry catheter as described in section 2.6.2.

3.2.2 Method:

Two healthy subjects attended fasted, having followed a low nitrate diet for 48 hours and gargled with 15mls of 0.12% chlorhexidine mouthwash bd for 48 hours prior to their visit. Initially a 2-channel (7cm apart) paediatric pH probe (26270, Medipius) was passed nasogastrically. This was withdrawn at 1cm increments every 1 minute to locate the pH step-up point. Confirmation of the step-up point was made following withdrawal of the distal pH probe. The pH probe was then removed. A manometry catheter (2281-R, Medipius, UK) was passed nasogastrically and after several minutes a SMPT was performed. Distilled water was used as the perfusate, and the water was delivered by a syringe driver via the lumen, as described in section 2.3.2 and Figure 2.5. This allowed a pressure profile of the LOS to be recorded and would confirm the location of the pH step-up to be within the LOS. The manometry catheter was then removed.

A segmented silastic tube of internal diameter 2.5mm and wall thickness 0.5mm (Altec, UK) was divided into sections measuring 16mm in length. A 180° degree clockwise twist was placed on the tube between the

segments. This twist was secured with a surgical silk tie – see Figure 3.1. The lumen of the segmented tube contained phosphate buffer solution at pH 7.4. A silastic tube was used as it allows NO to diffuse through it readily, akin to an epithelium. After passing through the silastic membrane the rise in pH causes the NO to react with the dissolved oxygen in the phosphate buffer, forming nitrite. This is then trapped within the segment. The ratio of nitrite formed from NO is 1:1.

Ten segments in total were created. This was attached to the manometry catheter with thin strips of Mefix tape (SCA Mölnlycke Ltd, Sweden). There is 5cm of dead-space distal to the 4 radial NO delivery channels on the manometry catheter. Segment number 3 of the tubing was placed with the NO delivery holes at its mid-point. Two sections were placed proximal to this. This meant that almost 10cm of segments would be below the level of the catheter. This is a relatively floppy structure, and for ease of intubation, a short section of a naso-gastric tube was taped to it, prior to insertion – see Figure 3.2.

Segmentation of the tube allows us to interpret the nitrite and NO profile exposed to the local environment for each individual 16mm segment. The fluid from each segment was extracted using a glass syringe. Each segment in turn was analysed by adding Greiss reagent and performing light absorption analysis (214). The nitrite concentrations would represent the profile of NO along the tube's length.

The manometry catheter with attached silastic and nasogastric tubes was then passed back into the subject via a nostril. The catheter was taped to the nose when the 4 NO delivery holes were at the level of the pH step-up point. The subject was then sat upright and the syringe driver, containing a

NO generating solution (see section 2.3.1 and Table 3.1), was commenced at the standard experimental flow-rate (99.9mls/hr) for 23 minutes. Taking into account the distilled water perfused through the dead-space, this is equivalent to 15 minutes of NO solution from the catheter ports. De-oxygenated distilled water was perfused through 2 proximal oesophageal ports (flow rate of 0.42mls/channel/minute) to match the conditions of the actual study.

3.2.3 Results:

The levels of nitrite detected varied slightly for each subject. Both subjects had little if any nitrite detected in the proximal two segments, followed by a rapid rise distal to this. Subject A had a maximum nitrite concentration of 294 μ mol/l in segment 4 compared to 76 μ mol/l in segment 4 for Subject B. Subject A's nitrite levels reduced rapidly over several distal segments, whereas the nitrite concentration of Subject B tailed off more gradually over the length of the silastic tube. A summary of the segmental nitrite concentrations can be seen in Table 3.2. A schematic histogram depicting the variation of nitrite concentration for Subject A, correlating to the LOS pressure profile, the NO delivery site and the pH step-up point, can be seen in Figure 3.3.

3.2.4 Discussion:

When NO was delivered via the 2281-R manometry catheter to the pH step-up point of a healthy subject, virtually no NO was detected above the

HPZ in the lower oesophagus. For Subject A the majority of the NO is found in segments 3 and 4, with a little in segment 5 as well. For this particular Subject this was almost exclusively in or at the lower border of the LOS. Subject B had a less intense exposure at the lower border of the sphincter and also had more nitrite detected further away from the sphincter. However, it has to be appreciated that each Subject's anatomy may be slightly different and it is not known if the tube is lying down a gastric fold where it is more likely to come into contact with a pool of the NO solution. Factors such as this will dictate the profile within the tube. Regardless of this, the segmented tube is useful for detecting NO present within the stomach. We have shown reasonable levels present after 20 minutes and not all in the distal stomach.

3.2.5 Conclusion:

With a slow infusion rate of NO solution there is no great amount of run-off into the distal stomach. This reassures us that any absence of an affect seen on the LOS, for the doses of NO used in the fasted study, cannot be attributed to the disappearance of NO into the distal stomach following its infusion. The single syringe driver and loom is an effective way to deliver a NO solution locally to the environment of the upper GI tract. Its delivery flow-rate allows a good local exposure when attempting to perfuse an NO rich solution to the gastric cardia environment of the LOS.

3.3: Assessment of luminal NO delivery profile in the stomach, from a 2282 manometry catheter following a meal.

3.3.1 Aim:

To assess the distribution of NO at the GOJ after a meal using a segmented silastic tube and a 2282 manometry catheter as a NO delivery system.

3.3.2 Method:

The same question was asked of the meal study as of the fasted study. Not only was the total delivery of NO greater over the test but also the catheter delivering it was different as well. Also, the flow rate of each test solution was greater per channel, and the concern was that there might be a greater opportunity for the solution to run off, away from the proximal stomach. The manometry catheter used for the meal experiment was a Mediplus 2282 model, which has perfusion ports at a different location to the 2281-R. The distal 4 channels, used for recording during the experiment, but also delivering a NO solution, are placed 1cm apart (ie: spanning 3cm). These channels were located with the most distal channel in the proximal stomach and the other 3 traversing the LOS.

The fasting experiment had 4 perfusion ports all at the same level, and within the LOS. This delivered a highly acidic solution. With shortening of the LOS occurring after a meal, the spacing of the ports on the 2282 catheter meant that the upper of the 4 distal ports may only be just within the LOS or

even in the lower oesophagus. Acid from this port would cause reflux contamination of the oesophagus. Therefore, hydrochloric acid was only included in the distal 2 ports (gastric channels). The proximal channels were referred to as the 'oesophageal channels'. Contents of the test solutions in the 4 channels can be seen in Table 3.3. As it is expected that an acid pocket would more than likely be formed after a meal, extending up into the distal LOS, locating a pre-prandial pH step-up point was not incorporated into the protocol.

Two healthy subjects attended fasted for this study. They had been on a low nitrate diet for the preceding 48 hours, and used chlorhexidine mouthwash for 48 hours, as previously described. A 2282 manometry catheter was passed nasogastrically and a SMPT of the fasting LOS performed. They were then asked to eat a battered fish and chip meal with a glass of still water. A post-prandial SMPT of the LOS was repeated. This allowed identification of the lower border of the LOS, just as would be done in the full study. A similar segmented silastic tube was attached to the side of the 2282 catheter using mefix tape. The most proximal tubing segment correlated to the most proximal of the 4 solution delivery channels. The distal of the 4 solution delivery channels correlated with the third tubing segment. This left 11.2cm of tubing extending past the distal delivery channel. Again, a short section of a naso-gastric tube was taped along side this to stiffen it and ease intubation – see Figure 3.4.

The tube was passed naso-gastrically and placed so that the distal delivery port was located just distal to the lower border of the post-prandial LOS, and within the proximal stomach. The tube was taped in position to the nose. With the subject in the upright position, the test solutions were run at

99.9mls/hr through the 4 syringe drivers for 20 minutes. Taking into account the dead-space of distilled water from the infusion, this is equivalent to 17 minutes of NO solution from the catheter ports. The 4 remaining proximal channels on the manometry catheter were perfused with de-oxygenated distilled water (flow rate 0.45mls/channel/minute), to match the conditions of the study. The tube was removed and the phosphate buffer solution removed from each segment in turn. Each was analysed individually for nitrite content.

3.3.3 Results:

As would be expected, with a slightly longer NO infusion time and also a greater volume of solution, the cumulative amount of nitrite seen in the tube segments is greater in this meal study than the fasting equivalent. The majority of the NO appears to be in the proximal stomach. However, there is also NO seen in the most proximal segment of both patients. This is at a level proximal to the NO delivery site. For Subject C the level of nitrite is slightly higher in the proximal segment (177 μ moles/litre) than in segment 2 (158 μ moles/litre). For Subject D the level is very much higher in segment 1 (306 μ moles/litre) than in segment 2 (147 μ moles/litre). A breakdown of the segmental nitrite results can be seen in Table 4. A schematic histogram of the nitrite profile in the tube for Subject C, with correlation of the post-prandial LOS pressure profile and the location of the manometry infusion ports can be seen in Figure 3.5.

3.3.4 Discussion:

There is clearly a reasonable amount of NO retained in the proximal part of the stomach. It is not running off directly to the distal stomach. More strikingly than this is the appearance of nitrite in the segments of the tube which are above those delivering the NO solution. Each segment is twisted and bound, to prevent leakage between segments, and reduce inter-segment contamination. Therefore, nitrite found in the silastic tube above the lower 2 ports is in fact NO that has reached there by means of reflux. The subjects had an erect posture for this protocol demonstrating reflux against gravity.

Comparing this to the previous work discussed would suggest two methods for the luminal appearance of NO seen in Barrett's patients following a nitrate meal (166). The NO could itself be refluxed from the proximal stomach, or hiatus hernia as described here. Alternatively, the salivary nitrite and thiocyanate travelling down the oesophagus could meet refluxed gastric acid and ascorbic acid and generate the NO within the lumen of the oesophagus, in a more proximal position than what happens with the normal chemistry.

3.3.5 Conclusion:

After 17 minutes, the NO, and subsequently the nitrite captured in the silastic tube, delivered by the 2282 manometry catheter following the meal appears to be retained in the proximal few centimetres of the stomach. This is almost in keeping with normal upper gastro-intestinal physiology. There is also evidence of probable NO reflux occurring into the oesophagus. This led us to examine this closer in the next experiment.

3.4 : Assessment of luminal NO delivery profile in the oesophagus, from a 2282 manometry catheter following a meal.

3.4.1 Aim:

To assess the distribution of NO in the oesophagus after a meal, using a segmented silastic tube and a 2282 manometry catheter as a NO delivery system.

3.4.2 Method:

The same protocol for the previous meal experiment was performed. Two healthy subjects were fasted and had followed a low-nitrate diet. They also used 0.12% chlorhexidine mouthwash bd, as described before. They were given a battered fish and chip meal with a glass of water to eat. A slow motorised post-prandial LOS pull-through was performed. This allowed the manometry catheter to be placed, as before, with the distal delivery channel in the proximal stomach. A segmented silastic tube was again attached to the manometry catheter, but this time with the segments extending up into the oesophagus. The middle of the distal segment of the silastic tube corresponded to the distal NO delivery lumen. Unlike experiments 3.2 & 3.3 of this series, no extension was added onto the end of the manometry catheter as the full length of the silastic tube ran within the limits of the catheter – see Figure 3.6.

Following the meal the catheter was placed naso-gastrically, in the location described previously. The NO solutions (see Table 3.3) were infused

for 20 minutes (flow rate 1.67mls/minute/channel), equivalent to a 17 minute NO exposure, with de-oxygenated distilled water perfusing the oesophageal ports (flow rate 0.45mls/channel/minute). After this, the assembly was removed. Extractions of the phosphate buffer were made with the glass syringe from each tubing segment. The nitrite levels in each segment were assessed using the Greiss reaction and compared to those of the previous experiments.

3.4.3 Results:

A different nitrite distribution was observed between the 2 subjects. Subject E has nitrite present as proximal as segment 3. Here it is present in low concentration (6 μ moles/litre). However, there are more significant concentrations present distally to this in segments 4 and 5 (90 and 80 μ moles/litre respectively). The pattern of NO exposure in Subject E shows greater levels on moving closer to the LOS. Subject F, however, has little evidence of NO exposure to the oesophagus with only 18 μ moles/litre measured in segment 8, just above the LOS, but a similar pattern of NO exposure within the LOS compared to Subject E. A schematic diagram of the nitrite levels for Subject E can be seen in Figure 3.7 and a comparison of the absolute nitrite levels for both Subjects E and F in Table 3.5.

3.4.4 Discussion:

These two studies show the potential variation in distribution of nitrite through the silastic tube. This equates directly to different NO exposures to

the tube, cardia and the oesophagus. Although the NO solution was only run for 17 minutes, both the amount of NO and the proximal extent of it's distribution is quite striking in Subject E. The level of proximal NO detection, in tube segment 3, is equivalent to 8cm above the upper border of the post-prandial LOS.

No pH probes were attached to the manometry catheter. It is likely that the NO profile reflects that of the reflux episodes and their degree of proximal extension. Taking this into account, these experiments also show how some people are more prone to reflux episodes after a meal. Despite a significant degree of reflux in the first experiment, Subject E was entirely asymptomatic. When the manometry catheter is attached to the silastic tube, the assembly can measure almost 7mm in diameter. Due to the helical arrangement of the manometry outlets on the catheter it is also difficult to avoid the silastic tube sitting in close proximity to several of them. This makes mucosal contact and channel outlet obstruction unreliable. For this reason manometry recordings were not made during this brief infusion. The distilled water running through the oesophageal ports was continued to mimic our standard experimental conditions. Once again, the actual concentration of the nitrite measured in the silastic tube was itself not terribly important, but the evidence of its distribution was.

Winter *et al* performed similar silastic tube experiments in 15 healthy subjects. A 21-segment tube was passed nasogastrically and placed across the GOJ. Comparisons were made of the nitrite concentrations in the segments after both a control (water) drink and a 2 millimole potassium nitrate drink. The tube was left *in situ* for approximately 2 hours following ingestion of nitrate. These experiments were performed in the fasting state.

Peak concentrations of nitrite (93.3-264 μ M), occurred following the nitrate meal, and appeared at the distal end of the LOS – Figure 3.8 (217). These are comparable to the 2 maximum levels seen in our manometry catheter fasting experiments of 294 μ M and 76 μ M (Section 3.2).

3.4.5 Conclusion:

From these experiments we can confirm that we have a delivery system for luminal NO, similar to that of upper gastro-intestinal physiology (217). Although, in our experiments, there were no pH probes present, we have seen evidence of the reflux of the lumenally delivered NO, by its presence as nitrite in the silastic tubing segments. Also, there were no formal control runs for our experiments. It should not detract from the compelling nature of the nitrite results. We have also shown the variability seen between healthy subjects. Despite their 'healthy' status they show differences in their profile of NO exposure in the oesophagus. The reasons behind this are complex. These are the issues which possibly decide who goes on to develop GORD and its consequences, and who doesn't. Furthermore, there are issues of variability in mucosal protection, separating those patients with GORD and those with non-erosive reflux disease (NERD). These mechanisms are not fully understood.

Table 3.1: Syringe contents for the fasting NO-generating study day.

Syringe concentrations
Thiocyanate – 1mM
Hydrochloric acid – pH 1.8
EDTA – 1mM
Nitrite – 1.6mM
Ascorbic Acid – 5mM

Table 3.2: Concentration of nitrite in segments of the silastic tube after a NO infusion from a 2281-R manometry catheter, in the fasted state. Section 1 is in the distal oesophagus, section 3 is at the level of the NO infusion and section 10 is in the mid-stomach.

Tube segment	Subject A	Subject B
	[Nitrite]/micromoles/litre	[Nitrite]/micromoles/litre
1	6	0
2	15	2
3 (LOS)	163	62
4	294	76
5	56	66
6	4	69
7	7	55
8	3	29
9	11	39
10 (Stomach)	8	26

Table 3.3: Syringe contents for the post-meal NO-generating study day.

Gastric channels/ Syringe concentrations	Oesophageal channels/ Syringe concentrations
Thiocyanate – 1mM	Thiocyanate – 1mM
Hydrochloric acid – pH 1.0	Nitrite – 1.6mM
EDTA – 1mM	
Nitrite – 1.6mM	
Ascorbic Acid – 5mM	

Table 3.4: Concentration of nitrite in segments of silastic tube following NO infusion after a meal and with a 2282 manometry catheter. Section 1 is in the distal oesophagus, section 3 is at the level of the NO infusion and section 10 is in the mid-stomach.

Tube segment	Subject C [Nitrite]/micromoles/litre	Subject D [Nitrite]/micromoles/litre
1	177	306
2	158	147
3 (LOS)	236	149
4	157	164
5	121	129
6	183	108
7	100	98
8	75	49
9	49	41
10 (Stomach)	39	31

Table 3.5: Concentration of nitrite in segments of silastic tube following NO infusion after a meal and with a 2282 manometry catheter. Section 1 is in the proximal oesophagus and section 10 is at the level of the distal NO infusion port.

Tube segment	Subject E	Subject F
	[Nitrite]/micromoles/litre	[Nitrite]/micromoles/litre
1 (Oesophagus)	0	0
2	0	0
3	6	0
4	90	0
5	80	0
6	116	0
7	180	0
8	144	13
9 (LOS)	100	156
10	104	122

Figure 3.1: A silastic tube segmented with surgical silk ties. Each segment contains phosphate buffer solution pH 7.4.

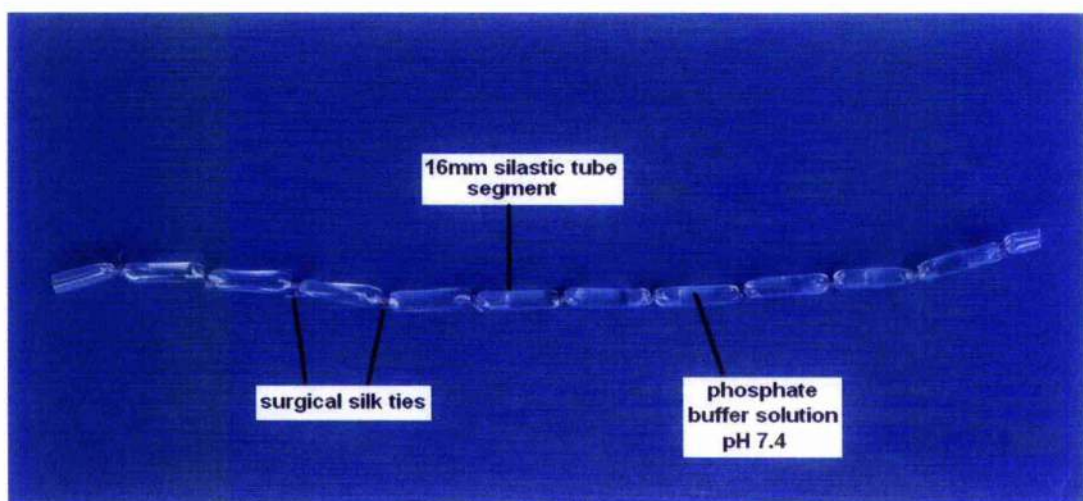


Figure 3.2: A segmented silastic tube attached to the 2281-R manometry catheter. Catheter used in the fasting study. Note the section of a nasogastric tube acting as a splint. Silastic tube will assess the NO profile of the fasted protocol at the GOJ and proximal stomach.

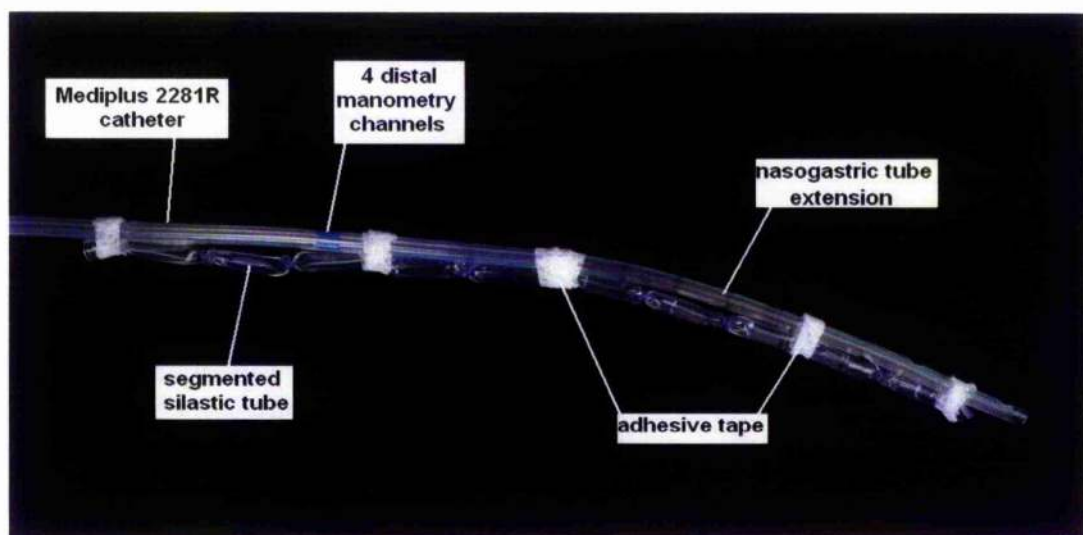
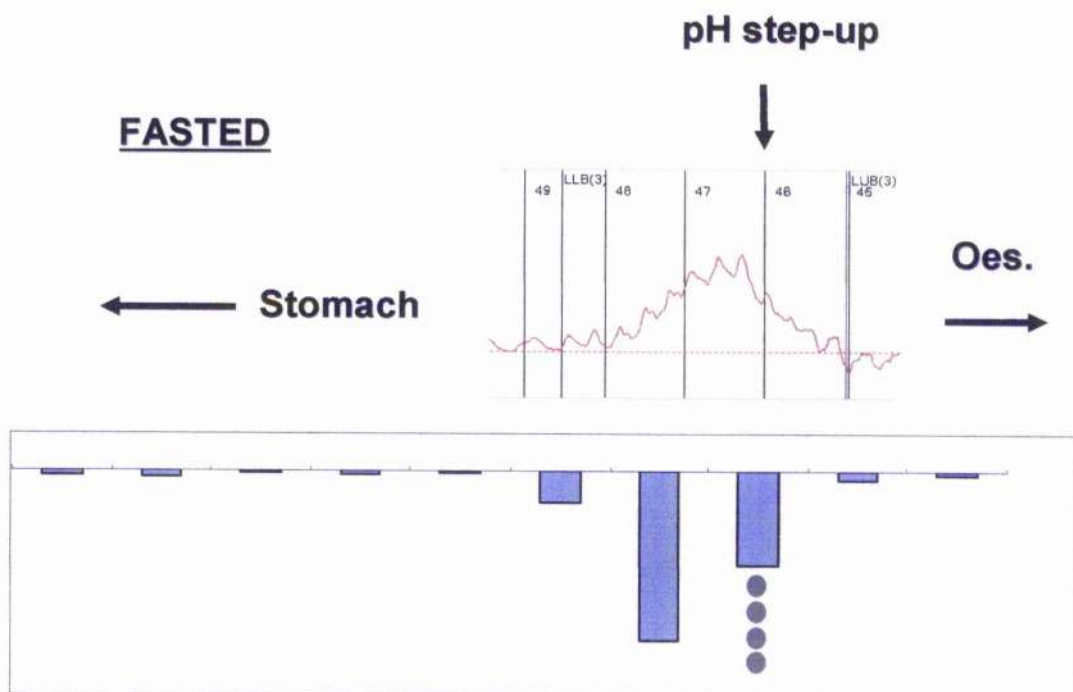


Figure 3.3: An example of Subject A's nitrite levels collected in the silastic tube after a 20 minute infusion of a NO generating solution (Table 3.1), with a fasted stomach. The 4 perfusion channels of the manometry catheter (Mediplus 2281-R) are represented by the ovals. Each segment measures 16mm in length. The segments' location relative to the LOS pressure profile, with an indication of the pH step-up point is shown. The histogram signifies the relative concentrations of nitrite compared to adjacent segments.



Key: Oes = oesophagus, LLB = Lower oesophageal sphincter lower border by manometry, LUB = Lower oesophageal sphincter upper border by manometry

Figure 3.4: A segmented silastic tube, attached to the 2282 manometry catheter, with a section of a nasogastric tube acting as a splint. This is used to assess the NO profile of the meal protocol NO infusion at the GOJ and proximal stomach.

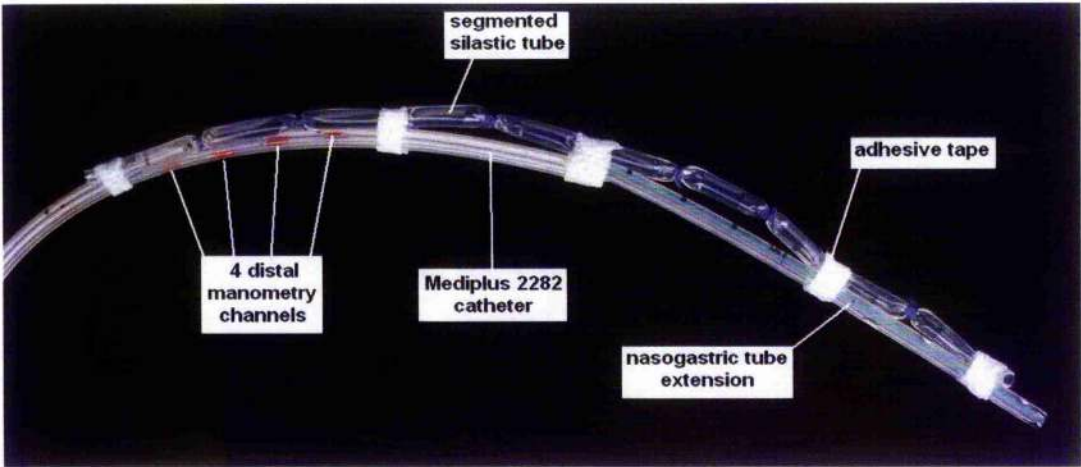
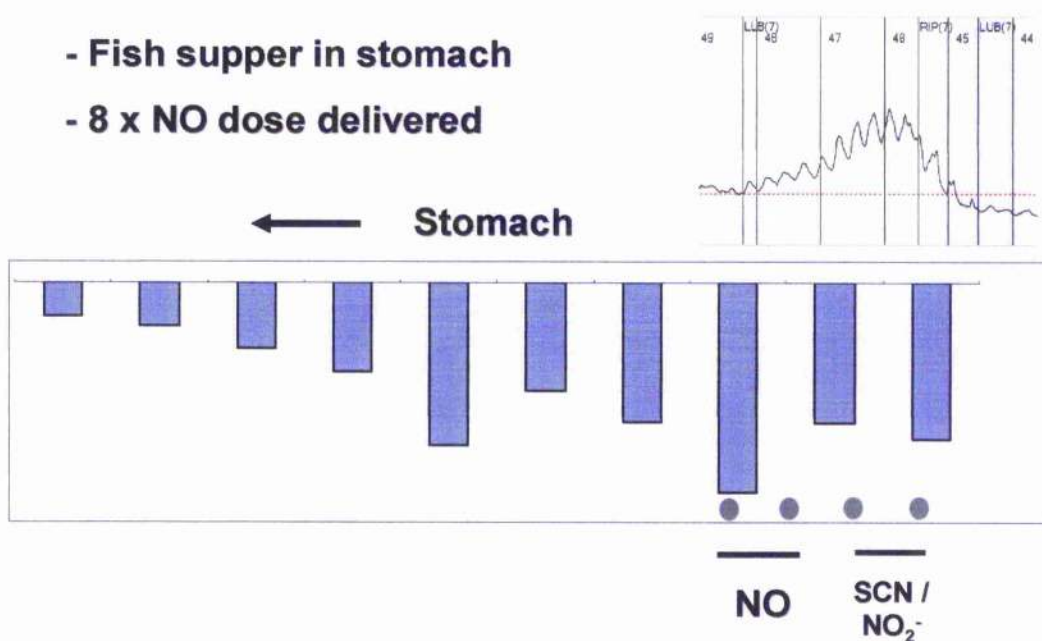


Figure 3.5: An example of Subject C's nitrite levels collected in the silastic tube after a 17 minute NO infusion. The 4 perfusion channels of the manometry catheter (Mediplus 2282) are represented by the ovals. Each is separated by 1cm. Only the 2 lower ports are generating NO, with the other 2 proximal channels containing nitrite (NO_2^-) and thiocyanate (SCN^-) – as per Table 3.3. There is an increase in the amount of NO delivered; 8-fold, compared to the fasting study. The location of the segments relative to the post-prandial LOS pressure profile is shown. The pH step-up point is not displayed as this changes location following a fish supper due to the proximal migration of the acid pocket. The histogram signifies the relative concentrations of nitrite compared to adjacent segments.



Key: LLB = Lower oesophageal sphincter lower border by manometry, LUB = Lower oesophageal sphincter upper border by manometry

Figure 3.6: A segmented silastic tube attached to the 2282 manometry catheter to detect NO oesophageal reflux following a meal. This is used to assess the NO profile of the meal protocol NO infusion at the GOJ and proximal oesophagus.

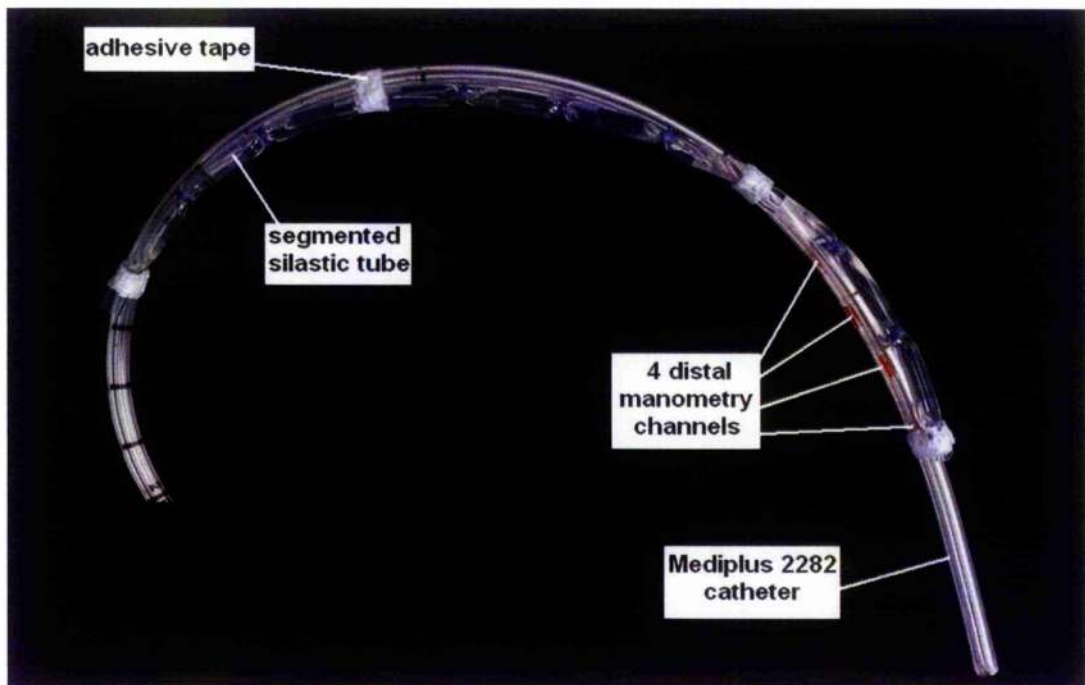
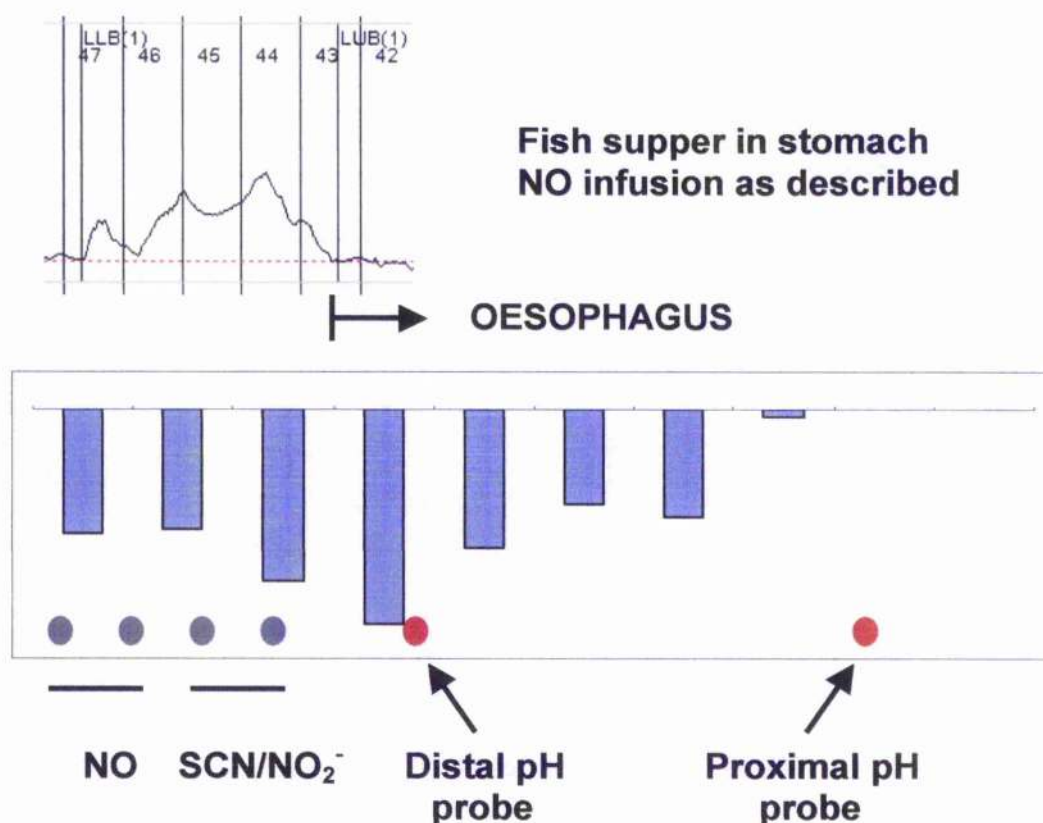
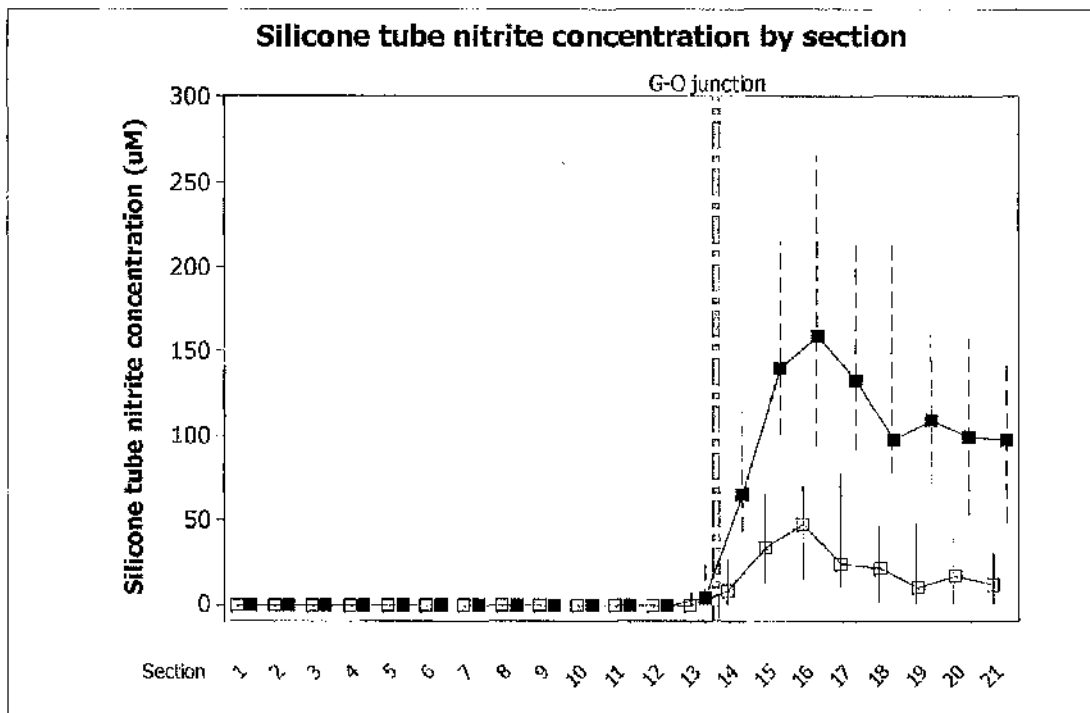


Figure 3.7: An example of Subject E's nitrite levels collected in the silastic tube after a 17 minute NO infusion. The 4 perfusion channels of the manometry catheter (Mediplus 2282) are represented by the ovals. Each channel is separated by 1cm. Solution infusions were identical to those of Figure 3.5 and Table 3.3. Each segment of silastic tube measures 16mm in length, and their location, in comparison to the post-prandial LOS pressure profile is shown. The standard position of the 2 oesophageal pH probes is illustrated with the red ovals. The histogram signifies the relative concentrations of nitrite compared to adjacent segments.



Key: LLB = Lower oesophageal sphincter lower border by manometry, LUB = Lower oesophageal sphincter upper border by manometry

Figure 3.8: Median silicone tube nitrite concentration by location for 15 healthy subjects. Sections 1-13 lie above the pH step up point and 14-21 below the pH step up point. Results following administration of the control drink are represented by (□) and following administration of 2 mmoles nitrate by (■). Nitrate meal peak level greater than Control ($p < 0.001$). Whiskers represent interquartile ranges.



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**Effects of luminal nitric oxide on the human
lower oesophageal sphincter, in the fasting state.**

Chapter 4

Chapter aims:

This set of experiments tests the hypothesis of luminal NO on the fasting LOS. Pull-through manometry of the LOS is performed before and after NO and control solutions have been infused into the cardia. MEEPs are compared as a measure of LOS pressure and statistical analysis performed on the results.

4.1 Introduction:

It is acknowledged that NO is present at high concentrations within the lumen of the upper gastrointestinal tract (218). In the presence of a healthy acid-secreting stomach the highest concentration is located at the gastric cardia (162). This correlates to the pH step-up point from the gastric columnar mucosa to the stratified squamous mucosa of the oesophagus. This location has been confirmed by x-ray studies with clips placed on the z-line (23). Manometry investigations have also shown that in healthy patients the z-line is positioned almost centrally within the high pressure zone of the LOS (15). The GOJ and lower oesophagus is becoming an area of increasing symptoms, disease and pathology throughout the Western world. The reflux of gastric contents is known to play a role in the inflammatory changes of oesophagitis and mucosal ulceration, the mucosal changes of Barrett's epithelium and even in the development of adenocarcinomas in this region (219;220).

The generation of NO is by a recycling process as previously described. 25% of nitrate absorbed from the diet or produced endogenously is taken up by the salivary glands and secreted into the mouth (221). Buccal bacteria convert a substantial proportion of the nitrate to nitrite. This results in a high concentration of nitrite in the mouth and throughout the length of the oesophagus for several hours after ingesting nitrate-containing foods (166). When the nitrite encounters acidic gastric juice, which contains vitamin C it is rapidly converted to NO.

Nitric oxide is well known as a messenger in cell signalling and smooth muscle relaxation. We know that NO is at its most concentrated at the cardia and that this is situated within the muscular high pressure zone of

the LOS. This led us to hypothesise that it may, in fact, have some effects on the motor control and performance of the LOS. Factors that predict the likelihood and severity of acid-reflux disease and reflux oesophagitis have been described (132). These include the resting LOS pressure, the total sphincter length and the intra-abdominal sphincter length. However, the actual mechanisms that lead to acid reflux are not well understood.

The luminal NO is normally generated in a region where columnar epithelium exists on top of an arrangement of longitudinal folds (37). Deep to this epithelium lies the muscularis mucosae whose muscle bundles are arranged longitudinally in the oesophagus (7). The thickness of the muscularis mucosae is greatly increased on top of the folds in the squamous-lined portion of the high pressure zone and this partly crosses into the columnar-lined sphincter (38). Distal to the sphincter the muscle bundles are arranged in a criss-cross fashion (222). Outwith the muscularis is the circular muscle layer, which is more substantial in the sphincter area than in the oesophagus, and then the longitudinal layer outside this completes the picture.

It is conceivable that the NO generated in the sphincter could cross the epithelium, as shown in our skin experiments (Figure 2.13). Even if it were not to reach the circular muscle, it may interact with and alter the physiology of the muscularis mucosae. It is assumed that the circular smooth muscle is the main component of the sphincter pressure and that the longitudinal smooth muscle fibres externally are there to control swallowing and peristalsis, but this is not proven.

This leaves a big question as to the function of the muscularis mucosae. Its anatomical location, distribution and orientation are such that it

is likely to have a specific role. Whether this is the case, and what role it may play, is speculative. The squamous epithelium overlying the thickened areas of muscularis mucosae related to the sphincter mucosal folds occasionally has a rimped effect giving the appearance of transverse folds (86). From a histological view it would seem that contraction of the longitudinal muscularis mucosae would be the cause of this. The benefits of such an action would be to bunch the mucosa up and plug a potential space where acid reflux may occur. Although, other schools of thought suggest that acid-reflux itself is the local stimulus to produce such an effect (91). Is it a secondary protective mechanism to prevent further acid exposure? The answer is unclear.

Therefore, does the muscularis mucosae also play a role in the prevention of acid reflux? Does it contribute to the resting tone of the lower oesophageal sphincter? If the answer to either of these is 'yes', then would luminal nitric oxide, which is likely to gain access to the muscularis mucosae, alter its role? If a resting tone exists in the muscularis mucosae, and this can be altered, this may then change the position of the overlying SCJ. Disease states of the oesophagus, including various degrees of oesophagitis and barrett's oesophagus, show differing locations of the SCJ, which are often fixed (15).

There is also a possibility that even transient longitudinal movements of the z-line, in either a cephalic or caudal direction, may play a major part in the increasing frequency of disease seen at this site. These episodes could be related to luminal NO exposure after a meal, and may become fixed over time. The same is true in the case of oesophageal shortening. This is a brief, natural phenomenon which occurs with swallowing (60). More prolonged episodes of shortening can be induced by exposure of the oesophageal

mucosa to acid conditions (73). Fluoroscopic techniques have shown this to be related to the muscularis propria (60), but no data is available as to the effect on the muscularis mucosae. If the oesophagus was to shorten, but a 'mobile' muscularis mucosae did not, then the z-line would descend into the acid environment of the stomach.

Clearance of acid from the oesophagus is impaired in patients with oesophagitis (223). This, in itself is enough to drive the perpetuating cycle of mucosal damage and increased cancer risk. Sildenafil (Viagra, Pfizer) is a phosphodiesterase subtype V inhibitor known to increase cGMP concentrations, the end result of NO's action, within the body, which is primarily used in male patients with erectile dysfunction. There is evidence in healthy volunteers, that increased levels of cGMP associated with sildenafil, play a role in impaired acid clearance (178). Although the NO origin of the cGMP rise in this instance is not intraluminal, it makes it all the more important to try and detect what effect luminal NO may have on the properties of the LOS and also oesophageal acid clearance.

The LOS is an incredibly complex and active structure. This could make it very easy to miss an effect from luminal NO, or even make it difficult to interpret a result. The best way to counter this is to break down the individual components of the sphincter and use both new and previously available techniques to address these in turn. We have developed a technique, which allows pressure measurements at the LOS, whilst delivering a controlled amount of externally generated NO to the lumen of the upper GI tract (see chapter 2.3).

Therefore, the main priority of this study would be to examine whether the presence of NO at the GOJ altered the resting pressure or tone of the

LOS. Although measuring changes in LOS pressure was the primary endpoint, other parameters would also be addressed.

4.2 Study participants:

This study was carried out in healthy subjects, who had no past history of upper gastrointestinal symptoms or surgery. It was confirmed that they were not taking acid suppression therapy or any other medication that could affect the motility or tone of the upper gastrointestinal tract. 12 subjects were investigated (6 male and 6 female) with an age range of 22 to 50 years and mean age of 32 years (Male mean age 28.5, range 22 to 42; Female mean age 35.3, range 23 to 50). The mean Body Mass Index (BMI) of the subjects was 25.3 with a range of 19.3 to 31.2 (Mean male BMI 24.3, range 19.3 to 31.2; Mean female BMI 26.3, range 21 to 30.4).

The *H.pylori* status of all participants was assessed by a urea breath test (C^{14}). Those found to be *H. pylori* positive were declined further involvement in the study. Pregnant or lactating females were also excluded. The participation of smokers was allowed, and their smoking status was documented. They were instructed not to smoke before attending on the morning of the study.

In the 24-48 hours prior to the investigation, the subjects were asked to follow dietary advice. This contained a list of 'dietary exclusions' in order to keep the diet low in ingested nitrates (224). To further reduce the possibility of salivary nitrate conversion to nitrite, the subjects were asked to use antibacterial mouthwash, containing Chlorhexidine Gluconate 0.12% (Chlorohex, Colgate Palmolive, UK). 15mls of the mouthwash was used to gargle and rinse the mouth for 1 minute on each of 3 occasions. This was done on the

morning and in the evening of the preceding day, and also on the morning of the investigation. The subjects attended after an overnight fast on the morning of their investigation.

4.3 Ethics:

This study was approved by the North Glasgow University NHS Trust Ethics Committee and all subjects gave written informed consent.

4.4 Materials & methods:

4.4.1 Manometry:

Water perfused oesophageal manometry was chosen to perform the pressure measurements during this study and also to act as a delivery system for our compounds to the mucosa in the LOS – as discussed in chapter 2.

4.4.2 Catheter:

An eight channel re-usable adult manometry catheter was used (2281R, Mediplus, UK). It was a standard 3.9mm diameter catheter with 5cm of dead-space distally. 4 channels were located at this point and arranged radially at 90° to each other. The other 4 channels were spaced at 5cm increments and in a helical fashion around the catheter, the most proximal being 20cm above the 4 radial channels. For the purpose of this study the 2 most-proximal channels of the manometry catheter were not used and were capped-off externally. There were several reasons for doing this. Firstly, we were mainly concerned with measuring the pressure of the LOS and so excessive information about oesophageal motility was not required.

Secondly, the 2 lower oesophageal recording channels were to be used as an indication of oesophageal peristalsis so that any form of LOS relaxation associated with these events could be discounted.

It was accepted that a submental proximal swallow sensor was not in place. This may make it difficult to differentiate between primary oesophageal peristalsis and the occurrences of secondary peristalsis following reflux episodes due to TLOSRS. It is known, however, that TLOSRS are very rare and infrequent in the fasting state (133). Finally, having only 2 channels proximal to the 4 radial channels, the volume of water running distally from here is reduced. This limits the theoretical dilution of drug at the LOS, giving a reduction in concentration but not total dose delivered. It also reduces the potential dilution of refluxed gastric juices, which may otherwise give an artificially high pH for the refluxate.

4.4.3 Pump devices:

Delivery of water through the manometry system was done in a novel manner. The 2 'oesophageal' ports were supplied by an electrically powered manometric pump with hydraulic water chamber (Mui Scientific, Model PIP-4-8); set to a chamber pressure of 10.5psi (70.2kPa). This pressure setting gave a flow rate of 18mls of water per channel per hour, which was found to be suitable at detecting oesophageal motility. The 4 radial channels were supplied by a 50ml syringe driver (Perfusor, B. Braun Medical Ltd, UK), at a flow rate of 25mls per channel per hour. The tubing arrangement and reproducibility testing data has been described in chapter 2.

4.4.4 Transducers:

Pressure changes were sensed by electrical pressure transducers (Synectics Medical Ltd, UK) located in series between the water infusion

devices and the manometry catheter, sampling at a frequency of 16Hz. During the study the transducers were situated at a level equivalent to the subject's stomach. The electrical signals from each channel were transmitted via a PVB transducer cable to a 12 channel Polygraph unit (Polygraf™ ID 12 Channel, Medtronic Functional Diagnostics A/S, Skovlunde, Denmark). The Polygraph was connected to a personal computer and the data collected and analysed using Polygram Net™ – Oesophageal Manometry software (Medtronic Functional Diagnostics A/S, Skovlunde, Denmark).

4.4.5 Manometry calibration:

A 2-point calibration was carried out for the 6 pressure channels. With water flowing through the channels at the stated rates. A zero reference point (equivalent to the stomach height during the examination) and a point 68cm above this were used. This height difference was used as 68cm, which is equivalent to 50mmHg. The default measurement scale for pressure on the Polygram Net™ software is mmHg.

4.4.6 pH probes:

For the purpose of this study a multi-use antimony crystal pH catheter was used. For the subject's comfort, ease of intubation and lack of distending properties to the oesophagus, a paediatric probe, 1.5mm in diameter, was selected. This was a dual channel model with the channels 7cm apart. The probe was equipped with an Ag/AgCl external reference electrode. The pH probe was attached to the manometry catheter with thin strips of adhesive tape. The lower recording channel was located 2cm above the 4 radial channels and the upper recording channel 9cm above the radial channels.

4.4.7 pH calibration:

Both the pH sensors and the external reference electrode underwent a 2-point calibration at room temperature, sampling at a frequency of 16Hz. Buffer solutions at pH 1.07 and pH 7.01 were used (Medtronic Functional Diagnostics A/S, Skovlunde, Denmark).

4.4.8 Solutions:

All solutions in this study were made with distilled water. One litre of distilled water was gassed with zero grade Argon (BOC Gases, UK) through a gassing 'stone' for 10 minutes to accomplish deoxygenation as described earlier (225). This was verified by a calibrated oxygen probe in the laboratory. This 'degassed' water was used to prime the tubing loom and also to fill the manometry reservoir. The manometry reservoir is pressurised by compressed air. Two factors would limit the amount of gaseous oxygen re-entering solution. Firstly the chamber was filled close to the top to reduce the amount of compressed air required to achieve the pressure. Secondly, the chamber is, even under normal conditions, equipped with a plastic float to reduce air bubbles in the water and form a seal on top of it, and this too reduces the amount of compressed air in contact with the degassed water.

As we had de-oxygenated the distilled water in the manometry loom, we approached the contents of the syringe in the same manner. The nitrite arm of the study is where the NO would be present in its greatest concentration and also where its' loss would be greatest. The rationale behind de-oxygenating the distilled water in the loom was that dissolved oxygen would mix with the NO solution on its way to the tip of the manometry catheter, and also potentially reduce its concentration by recycling.

The 2 test solutions to run, differed only by the inclusion of either nitrate or nitrite at a syringe concentration of 1.6 millimoles per litre. All other components were identical, including their final syringe concentration. They were Hydrochloric acid at a final pH of 1.8, Ascorbic Acid (Sigma, UK) 5 millimoles per litre, Sodium Thiocyanate (Sigma, UK) 1 millimole per litre and Ethylenediaminetetraacetic acid (Sigma, UK) 1 millimole per litre. Each of these was added at slightly different volumes to achieve the final 50ml syringe capacity, as outlined in Table 4.1.

Sodium nitrate or sodium nitrite from the stock solutions were the final syringe additions at 40% of the total volume. The reason for such a large volume compared to the thiocyanate and ascorbic acid was because we had previously noticed the formation of a brown gas on top of the solution when concentrated nitrite was added to acid. This was the formation of nitrogen dioxide (NO_2). This problem was rectified by altering both the concentration and volume of nitrite being added. Although this problem would not be encountered with sodium nitrate, we followed the same procedure, nonetheless, as the selection of nitrate or nitrite would be a blinded process. The hydrochloric acid was gassed with zero grade argon until deoxygenated, as was the sodium thiocyanate stock solution. The ascorbic acid was weighed out on a balance and also dissolved in deoxygenated distilled water.

It was not possible to gas the nitrite solution with Argon as it would reduce its total concentration. At pH 1.5 approximately 1% of nitrite exists as NO in equilibrium. At pH 7 (distilled water) less than 0.001% of nitrite exists as NO. This difference is due to a change in the equilibrium related to the pKa of nitrite. However, gassing a solution with Argon which contains NO, even in small quantities, would cause the NO to be lost to the atmosphere.

Once this has occurred the equilibrium is maintained by producing more NO from nitrite, and so over the period of gassing time a small, but significant amount of nitrite would be lost.

We used concentrated stock solutions of both nitrate and nitrite (100mM sodium nitrite dissolved in standard distilled water – not deoxygenated) and diluted this, 1 in 25 with deoxygenated distilled water. 20mls of this mix was used for the final syringe. Therefore, the 1ml of stock sodium nitrite solution was the only solution in the whole system with any remaining oxygen in it, and this was considered acceptable for the purposes of the study.

4.5 Protocol:

The subject was positioned on the examination table with legs outstretched in front with their upper body elevated at an angle of 45 degrees. In earlier pilot studies with the subject supine, when an acid containing solution was infused into the LOS, there was significant acid exposure to the oesophagus. This was presumably due to there being no control over which way the solution could flow because of its position in the sphincter. So, by sitting the subject up to 45 degrees, gravity could be used to preferentially direct the flow of the infusion. With the knowledge that TLOSRS are very rare in the fasting state we were satisfied that pressure recordings of the LOS could be made reliably during the resting phase and not during TLOSRS.

The external reference electrode was attached to the skin using electrode gel (Marquette Hellinge GmbH, Freiburg, Germany) to ensure good

contact, and a nostril was anaesthetised with lidocaine spray (Xylocaine Spray^R, Astra Zeneca AB, Sodertalje, Sweden). The tip of the manometry catheter and the pH probe was lubricated with a water-based lubricating jelly (K-Y Jelly, Johnson & Johnson, Sezanne, France) and passed through the nostril into the pharynx area. As much lubricant as possible was removed from the antimony crystals of the pH probe to ensure good recording. At this point the subject was asked to sip some water with a straw. In co-ordination with the swallows, the tube was passed into the oesophagus and through into the stomach.

Location of the assembly could be assessed by the distance markers along the side of the manometry catheter. Also, passage of the catheter from the neutral pH of the oesophagus to the acid of the stomach could be seen sequentially on each of the pH recording channels. At no point during the catheter insertion were any fluids being pumped through the manometry channels. The apparatus was left for several minutes to allow the patient to get used to the sensation of the catheter and also to allow more of the lubricant to be removed by the gastric mucosa and juices.

4.6 pH pull-through:

The assembly was withdrawn in 1cm increments each minute. A measurement was taken from the nostril at the point where the pH increased from acid ($\text{pH} < 2$) to neutral ($\text{pH} > 6$) (Figure 4.1). This location is consistent with the pH step-up point from acid to neutral which, in the fasted state, equates to the position of the SCJ. Once this position had been confirmed, the assembly was reinserted into the stomach, and all six channels of the

manometry system were perfused with de-oxygenated distilled water, by the pumping arrangement, as described earlier.

4.7 Sphincter pressure analysis:

Measurements of sphincter length, pressure and RIP were achieved using a SMPT of the manometry catheter. A motorised catheter puller was used (Medtronic Functional Diagnostics A/S, Skovlunde, Denmark), the activation of which could be controlled by the Polygram Net software. The fasting pressure pull-through was used to show the location of the pH step-up point in relation to the LOS, and by locating the RIP, it could also show its relationship to the hiatus (226). The catheter pull was started with all 4 radial channels just below the LOS and measuring gastric baseline pressure. Pulling for 10cm at a speed of 1mm/second gave a total pulling time of 1 minute 40 seconds. The subject was allowed to breath with normal shallow respiration. A pull-through was not started at least until a full minute after a swallow. These could be detected by visual observation of the subject or direct indication by the subject and confirmed by readings from the 2 oesophageal manometry channels, indicating oesophageal peristalsis.

The SMPT technique was used in preference to both a standard station pull-through and a rapid motorised pull-through. A standard station pull-through was not used as catheter withdrawals of 1cm or even 0.5cm increments were thought to be too inaccurate for measuring potentially small changes in LOS parameters. Even with the smaller catheter increment of 0.5cm, when calculating total sphincter length by the difference between its upper and lower borders, there is the potential for almost 1cm inaccuracy.

With a slow pull-through tracing on the Polygram Net software, markers are placed manually at the lower and upper borders of the LOS, and a time difference, in seconds, is displayed. With a constant pull speed of 1mm/sec, the LOS length can be calculated to the nearest millimetre. The reasons for not selecting a rapid catheter pull-through technique were that the RIP could not be identified and also there is known to be poor reliability with subjects' breath holding, which is a necessity for this type of pull-through. Also, if not able to identify the position of the RIP, it would mean that the calculation of 'abdominal' sphincter length would not be possible. Furthermore, a recent study has also shown that inter-observer and intra-observer variation is reduced significantly when analysing slow compared to rapid pull-through traces (49).

Of the six manometry channels, only the four radial channels were being used for the pull-through. So, the values given for HPZ length, RIP position, Abdominal Sphincter Length and MEEP are all taken as the average of four individual results.

4.8 Test solution infusion:

The manometry catheter was then re-inserted back into the stomach and withdrawn slowly to the pH step-up point (Figure 4.2). The assembly was taped to the nose and the infusion to the 4 radial channels was changed. The first syringe was pumped through the system for 28 minutes. During this time LOS pressure and oesophageal pH were recorded. The distal pH sensor was positioned just above the high pressure zone and the proximal channel 7cm above this, so that both short and medium segment reflux could be observed.

Bench top laboratory studies had shown that when the nitrite was present in the syringe to produce NO, it took approximately 8 minutes for the NO to be detectable at the catheter tip. The NO level then rises to a plateau at 10 minutes and then starts to decrease at 25 minutes (Figure 2.15C). Figure 4.3 also gives an example of this, and compares this to an exposure of a standard nitrate meal.

With the knowledge of the NO concentrations and delivery profile, it could be correlated to the trace when the NO exposure would be expected. Once the syringe contents had been delivered the catheter was immediately pushed back into the stomach. A SMPT, with the subject reclined at 45°, was repeated. Post-drug LOS parameters could then be collected.

4.9 Reproducibility:

Initial pilot studies to assess the effects of the nitrate or nitrite solutions on sphincter pressure had raised issues about reproducibility of results and interpretation of the traces. When assessing MEEPs, using a nitrate containing solution as a control, there was often a difference when comparing it to a NO generating solution. However, when each post-drug pressure was compared to the fasting gastric baseline pressure of the first SMPT with de-oxygenated distilled water from that particular study day, there was perhaps not as great a difference between these two. There also appeared to be a difference in the resting basal pressure of the sphincter on different study days. This would be addressed in the data analysis.

Therefore, in order to try and counter any problems with interpretation it was decided that the best way to approach the analysis was to undertake

two study days. The change was made to give both nitrate and nitrite containing syringes sequentially on each study day, but in a different order at each visit. This would allow direct comparison to the basal, distilled water pull-through and also to the other solution pull-through on that day. It would also allow for a cross-over analysis, to see if reversing the order of the two solutions had any effect as well. The nitrate and nitrite containers were randomised by a technician and labelled 'A' and 'B', to be unblinded at the end of the study.

At the end of the first syringes infusion, and at the time of the second pull-through, the second syringe was running through the four radial channels. Knowing that the solution took approximately 8 minutes to reach the tip, this gave a comfortable time period to perform the pull-through, which only took several minutes. There would be no possibility of contamination at the LOS by the second solution. After the second pull-through the catheter was re-introduced into the stomach and again withdrawn and secured, by taping to the nose, at the pH step-up point.

We have made the assumption that the pH step-up point (ie: SCJ) is at a constant location throughout the experiment, which is always in a fasting state. Any day to day variation in the location of the step-up point can be assessed by the initial pH pull-through profiles (Figure 4.4). A further SMPT was performed after the second syringe had been delivered. At the time of this third pull-through, de-oxygenated distilled water was flowing through the lumen, but again this would not have reached the distal channels by the end of the procedure.

When mixing the syringes, timing was quite critical. Solution 'A' or 'B' was always the last to be added to the syringe as NO generation was an immediate phenomenon. Also, we had observed that the NO level dropped in the syringe over time, so it was important that this was done at the last possible minute prior to infusion to achieve the highest concentrations in the catheter. Following the third pull-through, both pumps were switched off, the catheter assembly removed and the pH external reference electrode detached.

4.10 Trace analysis:

Data from the traces was analysed after each study. The fasting gastric baseline pressure was taken as the zero reference. Each of the traces was marked for the respiration cycle in which the pressure in that channel was persistently raised above the gastric baseline pressure, and this was marked as the lower border of the LOS. Another marker was placed for the respiration cycle where the pressure was equivalent to resting oesophageal pressure, and this was taken as the upper border of the LOS. The software then calculated the time, in seconds, between these 2 markers and this was then the equivalent sphincter length in millimetres. On the trace before the end expiratory pressure changed from a positive to a negative deflection, a separate marker was placed at the peak point of inspiration. This was classed as the RIP (Figure 4.5). The measurement of sphincter pressure was taken as the MEEP. This was recorded using a mobile vertical measurement tool. Mean values for each of the parameters were calculated from the 4 distal channels for that particular pull-through.

4.11 Comparison of results / statistics:

The issue of pull-through reproducibility had meant that the study design would allow various methods of results comparison. As far as we are aware, no one else has tried to deliver drugs or substances to the LOS by this type of set up, and neither have they tried to record parameters in this way. Creating a suitable control for this study meant that the only difference between 'A' and 'B' would be nitrate compared to nitrite. However, other studies have always compared tests to pull-throughs perfusing water. We are assuming that any changes seen in the LOS parameters are due to the infusion period of the drug and not due to any exposure during the pull-through process. We expect that it would be extremely unlikely to have such a rapid effect during a pull-through on the LOS. However, due to a short period lapsing between the end of the test infusion (1-2 minutes) and start of the pull-through recording the next series of data, a loss of effect from the solution could be a possibility. This is to be considered with NO having such a short half-life. However, the skin changes seen with our test solutions (Figure 2.13) were apparent for up to 5 minutes after wiping them off from the skin.

Analysing individual study days, the effects of both 'A' and 'B' can be compared to the initial water pull-through using a paired sample t-test. Then they can be compared to each other. Addressing the two study days together, 'A' data from one visit can be compared to 'B' data from another visit, which is what would have happened if the drugs were not run sequentially anyway. With both solutions being run on both days, this will give two sets of data. Variation caused by the order of the solutions on a

particular study day will also be assessed. As part of the investigation into reproducibility, a fasted resting sphincter MEEP analysis was performed. This compared the two initial water pull-throughs by calculating a correlation coefficient. Then, finally, both 'A's were compared to each other, and likewise both B's. The method of cross-over analysis is outlined in Figure 4.6. Not only can the LOS MEEPs be analysed in this way, but also the total sphincter length, the abdominal sphincter length and the position of the sphincter lower border.

4.12 Oesophageal acid exposure:

Further information on the exposure of the oesophagus to acid, for each experimental scenario, was analysed. Bed-side pH analysis required each tracing to be assessed by eye and each reflux episode documented for both channels. A pH less than 4 was classed as significant, as this has been widely used and accepted in all major publications to date, as the critical value for enzymic damage to the squamous mucosa. When added together, the sum of two infusions came to 56 minutes. However, due to the 'dead-space' run in of the tubing the first 7 minutes of recording had to be excluded. So, the pooled reflux events were for a total of 49 minutes. The 3 criteria assessed were a) the number of reflux events over the time period, b) the total time that the pH was below 4, and c) the mean duration of each reflux event. As described the lower pH recording channel was placed 2 cm above the radial infusion ports. This would position it either right at the upper end of the LOS or in the very lower part of the oesophagus. The second channel was 7cm proximal to this.

4.13 Results:

4.13.1 Lower oesophageal sphincter:

The study was completed by all of the 12 patients that enrolled. 24 full sets of interpretable data were obtained. Traces were analysed and the specific measurements taken as described and the solutions unblinded. Results for measured sphincter parameters were then grouped into either 'baseline' (Water), 'nitrate' or 'nitrite'. Each volunteer would, therefore, have results for 2 baseline, 2 nitrate and 2 nitrite pull-throughs. Due to the crossover design of the study the results for nitrite were pooled and likewise nitrate. Overall this gave 24 sets of data which were then compared using a 2-sided paired T-test. The MEEP, the highest pressure within the sphincter length, is used as a marker of the LOS pressure, and changes to this classed as the primary endpoint for the study.

Pooled results can be seen in Table 4.2 and are displayed in Figure 4.7. Furthermore, Tables 4.3 and 4.4 show the data split up to examine the effect of solution order. Similarly, results for sphincter length, abdominal sphincter length and sphincter lower border position were tabulated as sets of 24 pooled results, and then individually, in groups of 12, to examine any 'order' effects. These can be seen in Tables 4.5 and 4.6 and statistical analysis of these values in Table 4.7. The pooled results can be seen as paired line graphs in Figure 4.8.

4.13.2 LOS MEEP:

Comparing the nitrate (control) group (Mean MEEP 23.7mmHg, SD 12.3), to that of the nitrite group (Mean MEEP 23.9mmHg, SD 9.5), there is no significant difference between data sets. This applies to the data both

when it is pooled ($n=24$) to remove the effect of solution order ($p<0.96$), (Table 4.2, Figure 4.7) and also when it is subdivided into the groups ($n=12$) to particularly look for effects due to the order of which the solutions were delivered (Tables 4.3 & 4.4).

Although the baseline water pull-through values did not technically act as a control, statistical analysis was performed to see if we were dramatically changing the LOS physiology by undertaking the experiment. The results showed that the LOS pressure was not significantly different after our solutions than what would be expected after a normal pull-through where the catheter has water perfusing it. Pooled data gave p values <0.15 & <0.08 comparing baseline water pull-throughs to post-nitrate and post-nitrite respectively.

Addressing the issue of LOS pressure reproducibility, the day 1 baseline measurements were compared to those of day 2. Table 4.4 has already approached this in the form of a T-test, but by plotting one day against the other for the 12 volunteers a correlation co-efficient can be calculated - Figure 4.9. Perfect reproducibility from one day to another would be demonstrated by a correlation co-efficient of 1.0. A $Y=X$ line is plotted on the graph as a visual guide. The correlation co-efficient is calculated as 0.12, indicating relatively weak reproducibility. Looking at Figure 4.9 it can be seen that some of the pairs of subjects LOS pressures lie close to the $Y=X$ line, but some do show greater variability from day to day, leading to the low correlation co-efficient value.

4.13.3 Secondary LOS parameters:

We accepted that results of the other sphincter parameters would be classed as secondary observations. By this, we knew that if any analyses did reach statistical significance, then it could be related to chance. However, these would not be discredited, and may in fact give rise to further discussion points. The other parameters, total sphincter length (SL), abdominal sphincter length (ASL) and sphincter lower border position (SLBP) were also compared as pooled data ($n=24$) and as separate groups ($n=12$). As pooled data ($n=24$) there was no statistical difference between the nitrate or nitrite groups for these three variables (SL : $p<0.68$, ASL : $p<0.35$, SLBP : $p<0.11$). The same applied when the pooled nitrate and nitrite data was compared to the pooled water pull-through data, for the three variables. No group was statistically different from another (Table 4.5).

The same sub-group analysis ($n=12$) was performed for these three parameters. Analysis of the effects of the order of solution delivery as well as comparison to the water baseline results was performed (Tables 4.6 & 4.7). Virtually all of these results show no statistically significant difference. However, following the results seen for LOS MEEP, it was rather surprising to see that 2 of the 'p' values for Nitrite 2nd vs Nitrite 1st ($p<0.01$) and Nitrate 1st vs Nitrite 1st ($p<0.01$) reached statistical significance for the LOS lower border position. Mean values with 95% confidence interval for the pooled data can be seen in Table 4.8 and these are displayed as line graphs in Figure 4.10.

For the lower pH channel there was no statistical difference between nitrate and nitrite for any of the 3 criteria. However, there was a trend towards an increased number of reflux events (26.1 v 28.8, $p<0.49$), an increased

mean acid exposure time (1205 secs v 1351 secs, $p < 0.26$) and an increase in the mean duration of each event (96.5 secs v 186 secs, $p < 0.30$) for the nitrite (NO generating) group. For the upper pH channel the nitrite group had a statistically significant increase in the number of reflux events (4.41 v 6.2, $p < 0.02$) although, due to the low number of events, this is thought to have occurred by chance. There was a trend towards a longer acid exposure for the nitrite group, but this did not reach significance (566 secs v 630 secs, $p < 0.29$). The mean duration of each reflux event was slightly longer in the nitrate group, at the 9cm level, but this was not significant (62.3 secs v 60.8 secs, $p < 0.91$). Summary in Table 4.9.

4.14 Discussion and Conclusions:

As described, there was no effect seen on the LOS MEEP by the delivery of NO. There are several reasons why changes may not have occurred. It is likely that luminal NO simply does not have a marked effect at the GOJ with respect to resting pressure, in the fasting state. Although it may have been that an effect was too small for us to be able to detect it. NO can penetrate tissue as shown in our skin experiments. It is doubtful that NO is able to cross the tissues into the LOS circular muscle, not only due to its half-life, but also due to its affinity for haemoglobin in the rich blood supply of the submucosa. In the presence of oxygen and a more neutral pH environment, NO is converted first to nitrite and then to nitrate, rendering it irreversibly inactive. The speed of NO's reactivity *in vivo* and the fact that this occurs in immediately adjacent cells makes significant diffusion unlikely.

We considered a change in LOS pressure of 5mmHg as significant. Clearly this did not happen. In fact, in some patients, the pressure went up

after NO delivery. Dent *et al* described cyclical contractions of the human LOS in the interdigestive period (227). These occur during phase 3 gastric migratory motor complex activity. We were not able to ascertain if this was the case during this study as we were not concurrently measuring gastric pressure during the infusions or any of the pull-throughs. Therefore, we do not know where in this 'cycle' any of our pull-throughs have taken place. This also may well account for the day-to-day variation in baseline LOS pressure observed. The higher compliance of a syringe driver system with wide-bore tubing may also allow for both damping of the pressure signal as well as resonance in the fluid-line, which would be difficult to quantify. Experimental methodology could therefore be responsible for a 'missing' an effect.

It is also possible that after manoeuvring the catheter the effect of the solution may have worn off prior to the SMPT as described in section 4.11. We are also not entirely sure of the NO and nitrite chemistry at these high concentrations, and we are assuming equivalent chemistry above the levels where the reaction is certain. Despite these minor reservations, we expect the levels of NO to be somewhere between the two 'bench-top' curves and probably towards the higher concentrations.

The LOS is a very dynamic structure, which has fluctuating changes in its resting pressure from day to day, and hour to hour, as described above. This was demonstrated by the correlation co-efficient in Figure 4.9 of 0.12. This shows that there is weak reproducibility from day to day for a modest sized study group. Figure 4.9 shows that some of the patients fit quite close to the $Y=X$ line but 3 or 4 out of the 12 lie quite far from the line. Pre-study interviews confirmed that none of our patients were taking any medications

that would affect LOS pressure, and none of the smokers had smoked on the morning of the study (> 10 hours since last smoked).

The lumen at the GOJ boasts the highest recorded levels of physiological NO in the human body. At a cellular level this lipid soluble molecule is known to cross cell membranes, and cell layers in the vasculature. We have shown that NO is able to cross the human epidermis and relax the smooth muscle of pre-capillary arterioles (Figure 2.13). In this experiment there are also 3 other solutions applied to the forearm in parallel. Nitrite alone and also acidified thiocyanate with ascorbic acid are used as controls (Blebs 1 & 2 on the forearm accordingly). Bleb 3 comprised acidified nitrite without ascorbic acid. We know that this will not generate large amounts of NO. However, it is thought that in the GI tract after a meal, in the presence of thiol donors in food, or after interaction with Vitamin E on the epithelium's cell membranes, NO generating compounds may develop and cause smooth muscle relaxation or other intracellular effects by this route. Bleb 4 is the NO generating cocktail of all the aforementioned ingredients, and produces a significant erythema on the underside of the bleb. This was visible after 2 minutes, whilst the other blebs produced no significant results after 10 minutes. This erythema persists for up to 5 or 6 minutes after the solution is wiped from the skin.

We have also been able to detect NO on the serosal side of an *in vitro* rats stomach when the mucosal surface is exposed to an NO generating solution of similar concentration to that used in this study (unpublished work). There appears to be a degree of NO saturation in the tissue prior to reaching high levels on the serosal side. Traversing of NO across the tissues is thought to be more effective *in vitro* due to the absence of a mucous layer

and also an active intramural blood supply which could potentially decrease the amount of NO penetrating through to deeper structures.

Assessing the LOS in the fasting state is a very different situation to that accompanied by experimental gastric distension or the post-prandial state. After a fatty meal the LOS total length is reduced, with a preferential loss of the abdominal portion. After a fatty meal it has been shown that the pH step-up point migrates proximally, to a position above the SCJ (23). There is also a prising open of the mucosal rosette in the proximal stomach, which is thought to be one of the protective barriers against reflux. Mechanisms behind this are considered to be both mechanical, comparable to the neck of a balloon shortening as it inflates, and also due to circulating hormonal factors such as cholecystokinin, released by both gastric distension and constituents of the meal.

In the fasting state, the LOS is therefore a tightly plugged hole and it is only under a more stressed situation, such as after a meal, that reflux events are more likely. We know that mucosal folds exist through the length of the LOS lumen. In this 'bunched-up' arrangement, the distance for NO to travel to reach the underlying structures theoretically increases. After a meal, or following distension, one would expect the bulky mucosal folds to lessen, thereby thinning out and presenting less of a barrier to NO diffusion.

It is useful to consider what actually generates the pressure that we measure at the GOJ. Two major factors, namely the external circular muscle of the GOJ and the diaphragmatic crura are known to exert radial pressure, which protects the integrity of the LOS. Little has been said of the role played by the mucosal folds in the ability to exert pressure. Also, what controls the way in which these folds are arranged? They appear to be consistent in their

location as proved by the distribution of early tongues of oesophagitis sitting on the top of folds.

Do the folds represent regions of relaxed surface tissue that are passively concertinaed following contraction of the external structures, or do they have inherent tone which represents a third component to the LOS pressure? If the latter is correct, then the muscularis mucosae must be responsible for this. Again, the tone may be low and outwith the sensitivities of our equipment, but if the folds were put into a relaxed state, this may allow for subtle, but important changes to the reflux barrier. NO generated in this region would be more likely to have an effect on the sphincter function, but would not manifest itself as a gross reduction in LOS pressure.

It would be interesting to know what would happen if the mucosa in the LOS was stripped away. Would there still be a visible lumen previously occupied by the mucosa or would the circular muscle and crura obliterate this? Of course, this is very difficult to achieve in practice, and even in an animal model, trauma to the oesophagus and the physiological sequelae would make results almost uninterpretable in the *in vivo* setting.

The reflux profiles measured at the distal pH probe showed no difference between the nitrite and nitrate groups. GORD is multifactorial. Namely, the presence of a hiatus hernia, reduced LOS tone, the frequency of TLOSRS and impaired oesophageal acid clearance. Neuronal generation of NO is key in the last three factors. NO in this setting is derived from L-arginine and stimulates intracellular soluble guanylate cyclase to form cyclic GMP from GTP. Cyclic GMP causes intracellular phosphorylation, inhibiting smooth muscle contraction.

We have demonstrated the presence of NO in the lumen of the oesophagus following a reflux episode. It is not known how this luminal NO may alter acid clearance. The hypothesis would be that if it were to have any effect, then it should be inhibitory. Following this study, there is evidence for the lack of an effect, on the circular smooth muscle of the LOS, in the presence of modest doses of luminal NO. If NO were to penetrate at this site, it would very likely play a part. However, in the oesophagus, the longitudinally arranged muscularis mucosae, would be the first met and reachable target. What is the role of the oesophageal muscularis mucosae in acid clearance? The answer is simply that we do not know. Raising the intracellular cyclic GMP levels by oral sildenafil causes abolition of lower oesophageal peristalsis (178). Whether this could occur with luminal NO has not been addressed by any groups as yet. This is true for both healthy subjects and also Barrett's patients with a columnar-lined oesophagus, and a potentially different diffusion pattern of NO.

There has been recent interest into 'short segment' reflux of the oesophagus. Standard ambulatory pH monitoring places a single pH probe at 5cm above the LOS. However, although there may be a normal acid exposure at the 5cm level, there is often a potentially pathological exposure at the 2cm level (228). In healthy volunteers this pH drop is often imperceptible. This region is a common site of intestinal metaplasia in asymptomatic subjects (229), and the probable precursor for cancers here. Cancer of the gastric cardia is well established as not having any relationship with reflux symptoms (230). Clearly, if NO, which seems the most likely

agent, were to be responsible for short segment reflux, rather than standard reflux, then this is a major factor that could be addressed in cancer formation.

It is well accepted that both nitrite and nitrate can form carcinogenic compounds (231). However, it is not known what role, if any, NO plays in the formation of the post-prandial acid pocket. Although not directly addressing this, our subsequent studies detailed here will deal with the effect of luminal nitrite on gastro-oesophageal motility after a meal. There will also be a focus on both short segment reflux and reflux of a more standard nature.

Table 4.1: Stock solutions relative to the constituents of the 50ml syringes for both the Control and NO generating solutions.

Control solution / Nitrate				Nitric Oxide generating solution / Nitrite			
	[Stock solution]	Volume added	[Final syringe]		[Stock solution]	Volume added	[Final syringe]
HCl/EDTA	pH1.5/2.5mM	20mls	pH1.8/1mM	HCl/EDTA	pH1.5/2.5mM	20mls	pH1.8/1mM
Ascorbic Acid	50mM	5mls	5mM	Ascorbic Acid	50mM	5mls	5mM
Thiocyanate	10mM	5mls	1mM	Thiocyanate	10mM	5mls	1mM
Nitrate	4mM	20mls	1.6mM	Nitrite	4mM	20mls	1.6mM

Table 4.2: Comparison of lower oesophageal sphincter (LOS), maximum end expiratory pressures (MEEP), after nitrate (control) and nitrite (NO generating). Pooled data for 12 patients from both study days (n=24). Analysis by 2-sided paired sample t-test. Comparison by t-test to the pooled baseline water pull-throughs.

LOS MEEP / mmHg	Post-Nitrate	Post-Nitrite	p value <
Mean	23.7	23.9	0.96
Range	9.0–58.0	10.3–48.3	
Standard Deviation	12.3	9.5	
p value vs Pooled Water Data	0.15	0.08	

Table 4.3: Table of LOS Maximum End Expiratory Pressures (MEEP) after each of the six protocol pull-throughs (n=12).

LOS MEEP / mmHg	Water 1	Water 2	Nitrate 1	Nitrate 2	Nitrite 1	Nitrite 2
Mean	18.3	21.4	23.6	23.7	23.5	24.1
Range	8.7-35.5	9.9-38.9	9.0-58.0	11.4-47.6	12.8-39.8	11.0-48.3
Standard Deviation	8.28	9.28	14.93	9.69	7.54	11.55

Key:

1: First study day pull-through data 2: Second study day pull-through data

Table 4.4: Analysis of the effect of solution order (1st or 2nd) on the LOS MEEP, comparing both nitrate controls, both nitrite results and both baselines (Water pull-throughs). p value calculated using a 2-sided paired sample t-test.

Categories compared (n=12 each group)	p value <
Water 1 v Water 2	0.39
Water 1 v Nitrate 1 st	0.18
Water 1 v Nitrite 2 nd	0.15
Water 2 v Nitrate 2 nd	0.54
Water 2 v Nitrite 1 st	0.37
Nitrate 1 st v Nitrite 2 nd	0.98
Nitrate 1 st v Nitrate 2 nd	0.99
Nitrite 1 st v Nitrite 2 nd	0.87
Nitrate 2 nd v Nitrite 1 st	0.90
Nitrate 1 st v Nitrite 1 st	0.90
Nitrate 2 nd v Nitrite 2 nd	0.95

Key:

1: First study day pull-through data 2: Second study day pull-through data

Table 4.5: Pooled data of other measured LOS parameters.

n = 24	LOS length / cm			LOS abdominal length / cm			LOS lower border / cm from nostril		
	Mean	Range	S.D.	Mean	Range	S.D.	Mean	Range	S.D.
Water	3.93	2.6-5.9	0.78	2.73	0.6-4.8	0.95	46.89	39.1-50.0	2.03
Nitrate	4.22	2.6-5.9	0.94	2.87	1.2-4.7	0.94	47.19	40.8-51.6	2.63
Nitrite	4.15	3.1-5.2	0.67	2.65	1.6-4.3	0.76	46.66	39.1-49.8	2.88
Nitrate v Nitrite.									
p value <		0.68			0.35			0.11	
Water v Nitrate.									
p value <		0.12			0.56			0.10	
Water v Nitrite.									
p value <		0.24			0.73			0.49	

Table 4.6: Analysis of sphincter parameters related to their order and day of delivery. Day 1 nitrate solution was given before nitrite and vice versa on day 2.

n = 12	LOS length / cm			LOS abdominal length / cm			LOS lower border / cm from nostril		
	Mean	Range	S.D.	Mean	Range	S.D.	Mean	Range	S.D.
Water 1	3.80	3.2-4.7	0.58	2.66	1.4-3.8	0.81	46.9	39.1-50.0	3.08
Water 2	4.07	2.6-5.9	0.96	2.80	0.6-4.8	1.10	46.9	39.7-49.4	2.69
Nitrate 1	4.22	2.6-5.9	1.10	2.85	1.4-4.6	0.90	47.4	40.8-51.6	2.94
Nitrate 2	4.21	3.3-5.5	0.79	2.86	1.2-4.7	1.02	47.0	41.6-50.1	2.38
Nitrite 1	4.26	3.2-5.2	0.72	2.79	1.8-3.9	0.78	47.3	40.6-49.0	2.54
Nitrite 2	4.04	3.1-5.2	0.62	2.50	1.6-4.3	0.74	46.0	39.1-49.7	3.15

Table 4.7: Statistical comparison of the LOS parameters using a 2-sided paired sample t-test.

n = 12 each group	LOS length / cm	LOS abdominal length / cm	LOS lower border / cm from nostril
	p value <	p value <	p value <
Water 1 v Water 2	0.46	0.77	0.89
Water 1 v Nitrate 1 st	0.20	0.56	0.21
Water 1 v Nitrite 2 nd	0.08	0.73	0.13
Water 2 v Nitrate 2 nd	0.40	0.84	0.61
Water 2 v Nitrite 1 st	0.95	0.35	0.11
Nitrate 1 st v Nitrite 2 nd	0.92	0.83	0.87
Nitrate 1 st v Nitrate 2 nd	0.98	0.98	0.29
Nitrite 2 nd v Nitrite 1 st	0.46	0.18	0.01
Nitrate 2 nd v Nitrite 1 st	0.51	0.32	0.09
Nitrate 1 st v Nitrite 1 st	0.58	0.29	0.01
Nitrate 2 nd v Nitrite 2 nd	0.90	0.87	0.33

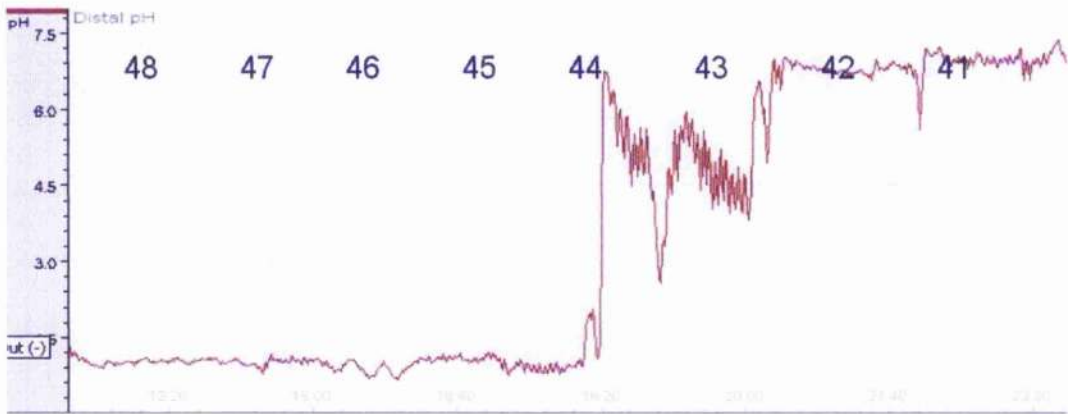
Table 4.8: Table of mean values with 95% confidence intervals (CI) for the data sets.

	LOS Pressure		Sphincter		Abdominal Sphincter		Sphincter Lower Border /	
	/ mmHg		Length / cm		Length / cm		cm from nostril	
	Mean	CI	Mean	CI	Mean	CI	Mean	CI
Pooled Water	19.9	2.98-37.3	3.9	2.4-5.5	2.7	0.8-4.6	46.9	41.2-52.6
Water Day 1	18.3	1.8-34.9	3.8	2.7-4.9	2.7	1.0-4.3	46.9	40.8-53.1
Water Day 2	21.4	2.8-39.9	4.0	2.1-6.0	2.8	0.6-5.0	48.9	41.5-52.2
Pooled Nitrate	23.7	-0.9-48.3	4.2	2.3-6.1	2.9	1.0-4.7	47.2	41.9-52.4
Nitrate 1 st	23.6	-6.2-53.5	4.2	2.0-6.4	2.9	1.1-4.6	47.4	41.5-53.3
Nitrate 2 nd	23.7	4.3-43.1	4.2	2.6-5.8	2.9	0.8-4.9	47.0	42.2-51.8
Pooled Nitrite	23.8	4.8-42.9	4.1	2.8-5.5	2.6	1.1-4.2	46.7	40.9-52.4
Nitrite 1 st	24.2	1.1-47.3	4.0	2.8-5.3	2.5	1.0-4.0	46.0	39.7-52.3
Nitrite 2 nd	23.5	8.4-38.6	4.3	2.8-5.7	2.8	1.2-4.4	47.3	42.2-52.4

Table 4.9: Reflux episodes at the 2 pH detector sites – 2cm and 9cm above the solution delivery site. Number of events was analysed using paired, 2-sided t-tests. Total time and mean duration were analysed using unpaired, 2-sided t-tests.

	2cm	2cm	p value <	9cm	9cm	p value <
	Nitrate Mean (SD)	Nitrite Mean (SD)		Nitrate Mean (SD)	Nitrite Mean (SD)	
Number of events	26.1 (17.3)	28.8 (25.6)	0.49	4.41 (6.3)	6.2 (6.1)	0.02
Total time pH<4 / secs	1205 (938)	1351 (996)	0.26	566 (863)	630 (827)	0.29
Mean duration of event / secs	96.5 (188)	186 (472)	0.30	62.3 (84.3)	60.8 (63.0)	0.91

Figure 4.1: Example of pH step-up at squamo-columnar junction.



Key: numbers along top represent cm markers from the nares. pH on y axis. Time on x-axis in minutes.

Figure 4.2: Location of the Four Radial Manometry Channels (2281-R catheter, Mediplus) in the LOS and position of the 2 pH recording probes.

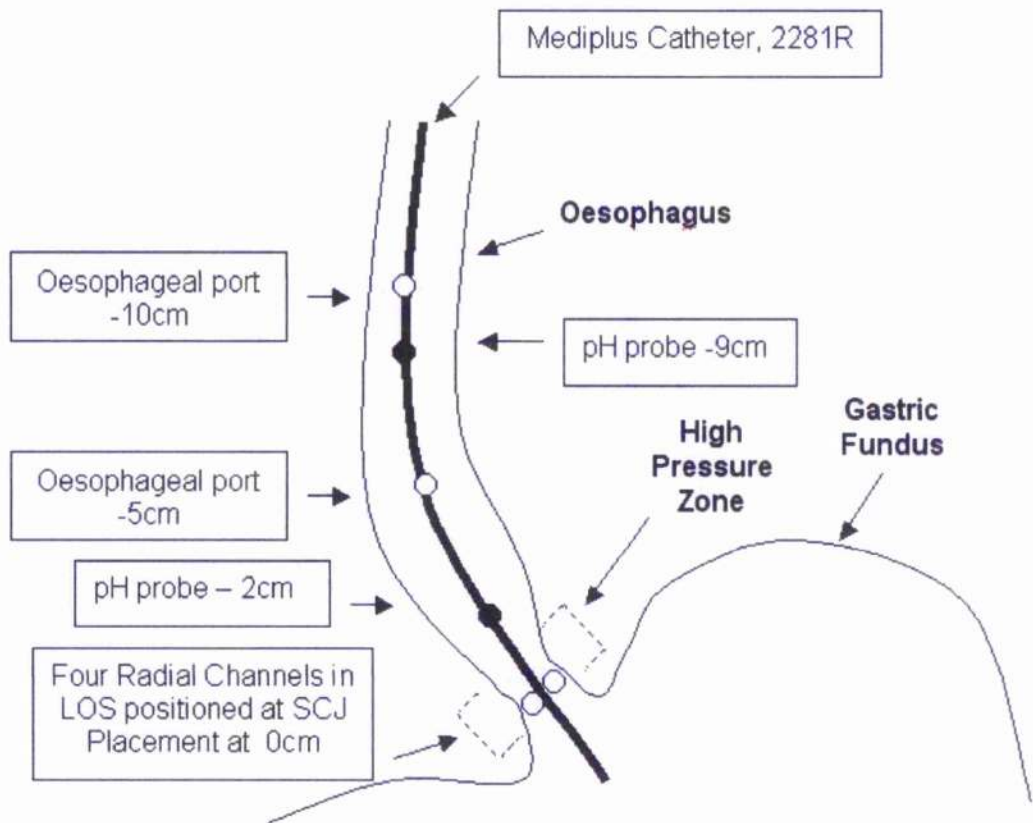
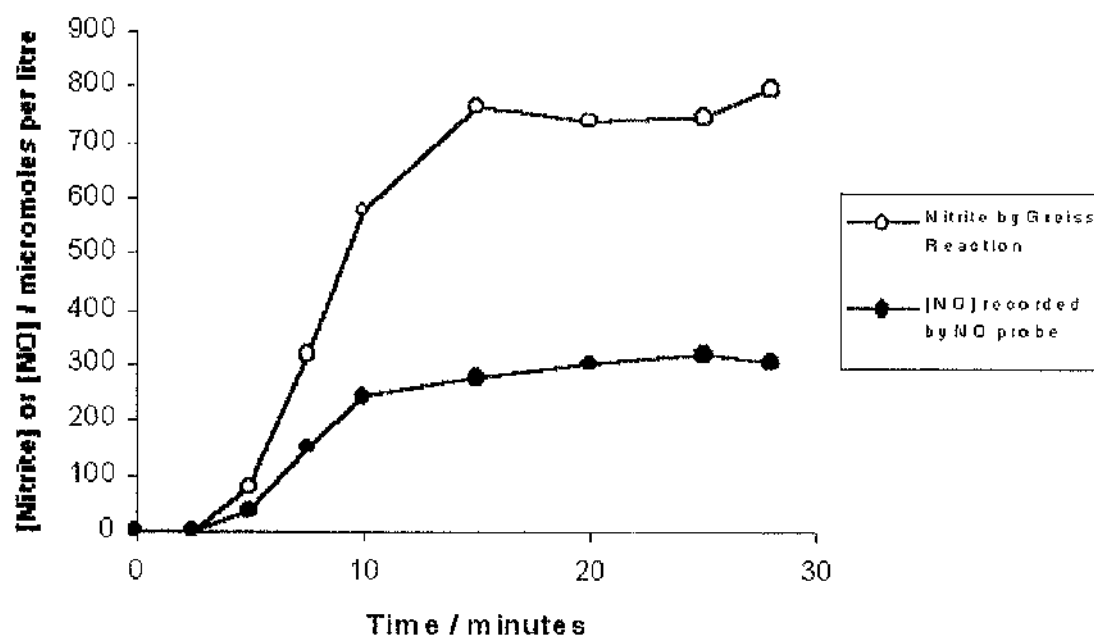


Figure 4.3: Graph to show concentrations of NO converted to nitrite measured by capturing the catheter's solution (Mediplus 2281-R) under phosphate buffer solution (pH 7.4) and then analysed by the Greiss reaction. Also, for comparison, the [NO] recorded directly by a bench-top NO probe (ISO-NO Mark II, World Precision Instruments) from the catheter tip.



Standard Nitrate meal of 2 millimoles KNO_3

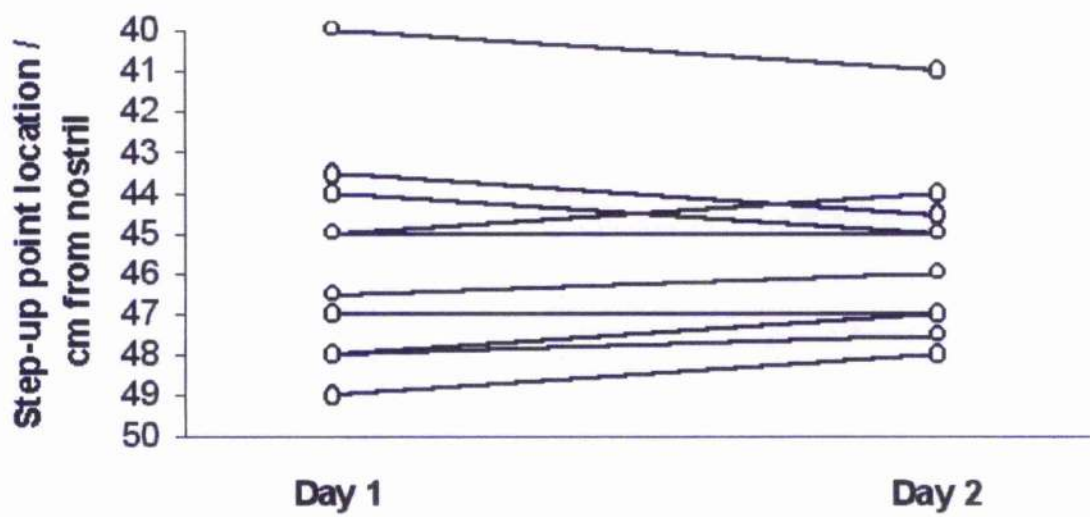
Expected to recycle 25% to salivary glands

Average conversion of 25% to nitrite in oral cavity by buccal bacteria

Therefore, $1/16^{\text{th}}$ of meal ends up as nitrite over 3-4 hours

Potential conversion to NO (Amount of Nitrite) = 125 μmoles

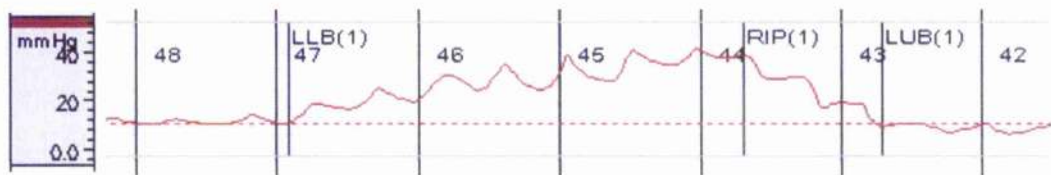
Figure 4.4: Day to day comparison of pH step-up points for the 12 volunteers.



Minor differences can be seen between visits, but are within the expected errors of the procedure protocol.

Figure 4.5: Manometry tracing depicting how the sphincter Lower Border, Respiratory Inversion Point and the Upper Border can be cited.

Slow motorised pull-through of LOS with syringe driver supplying radial channels.



Scale in mmHg. One centimetre pull-through markers on trace. LLB = Lower oesophageal sphincter Lower Border. RIP = Respiratory Inversion Point. LUB = Lower oesophageal sphincter Upper Border. Horizontal hatched line represents gastric baseline pressure.

Figure 4.6: Diagram to show how the cross-over analysis will work. Data from each of the 3 pull-throughs (water - baseline, A – post-nitrate & B – post-nitrite) from both study days (6 pull-throughs in total) can be compared to those on that day and also from their other visit.

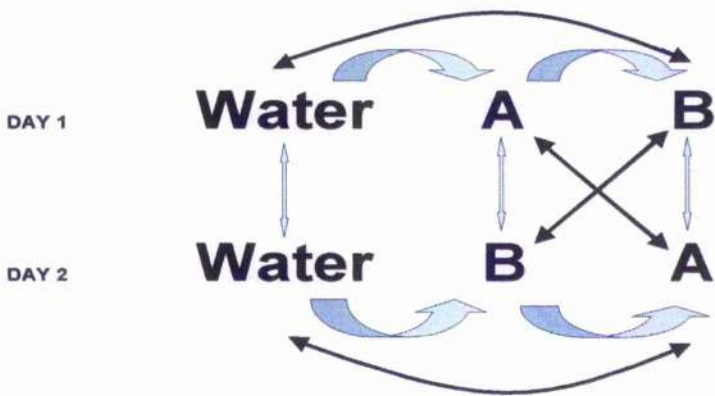


Figure 4.7: Comparison of the pooled data for the 12 volunteers. LOS MEEP/Pressure post nitrate and nitrite. n=24 for each group. p value < 0.96.

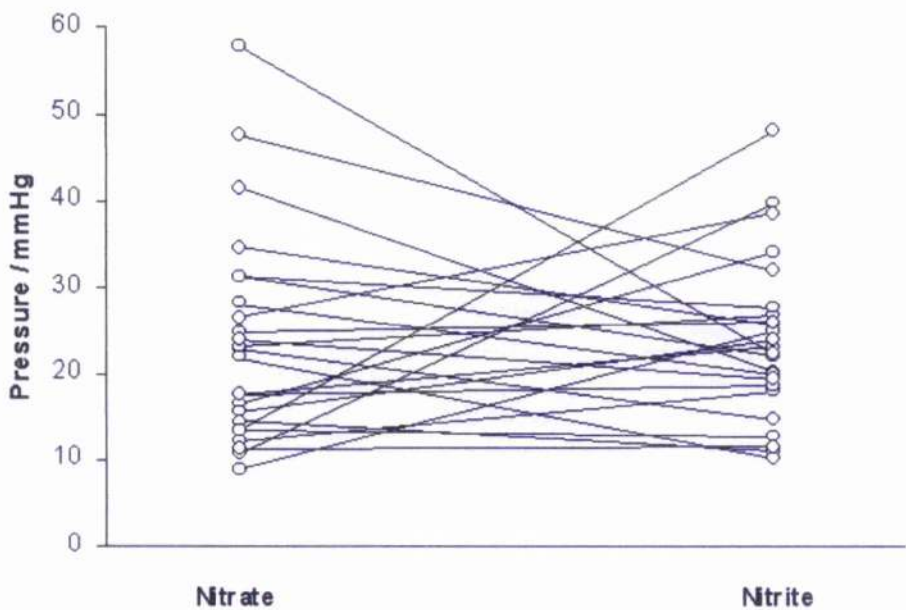


Figure 4.8: Paired data for the pooled results of the other LOS parameters.

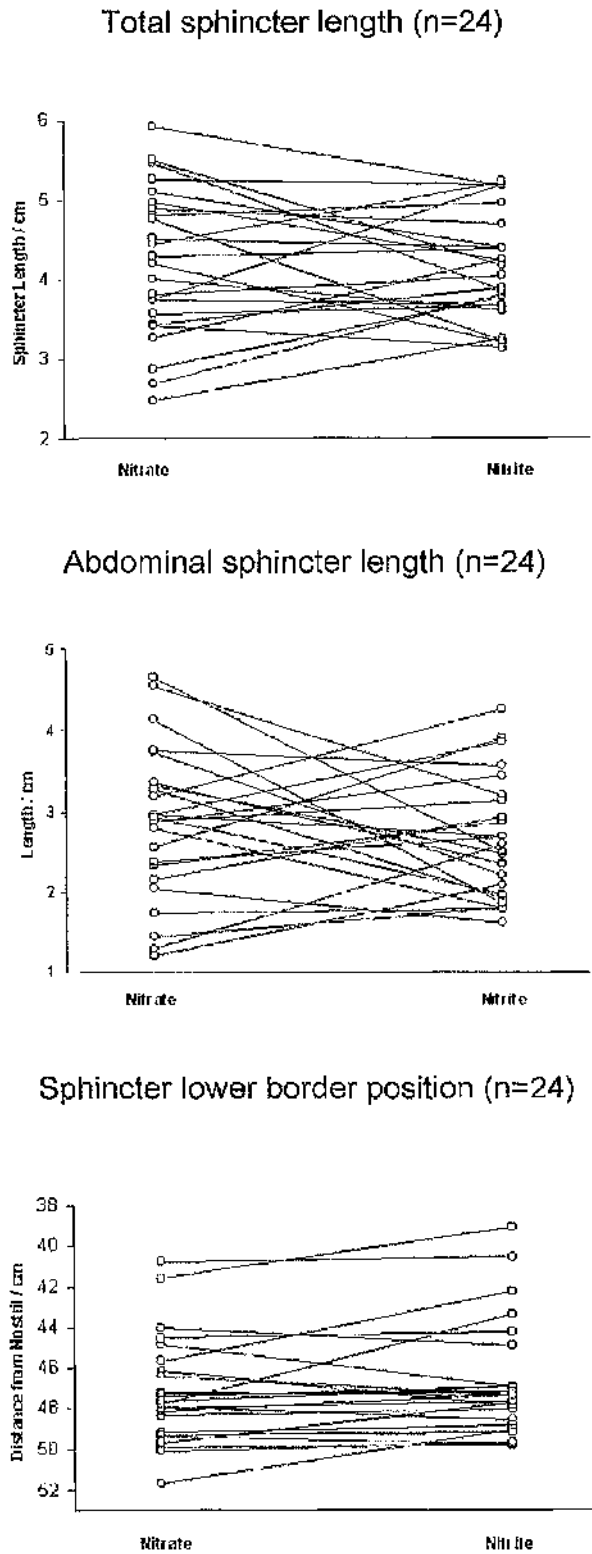


Figure 4.9: Reproducibility graph of LOS MEEP (n=12). Correlation coefficient = 0.12.

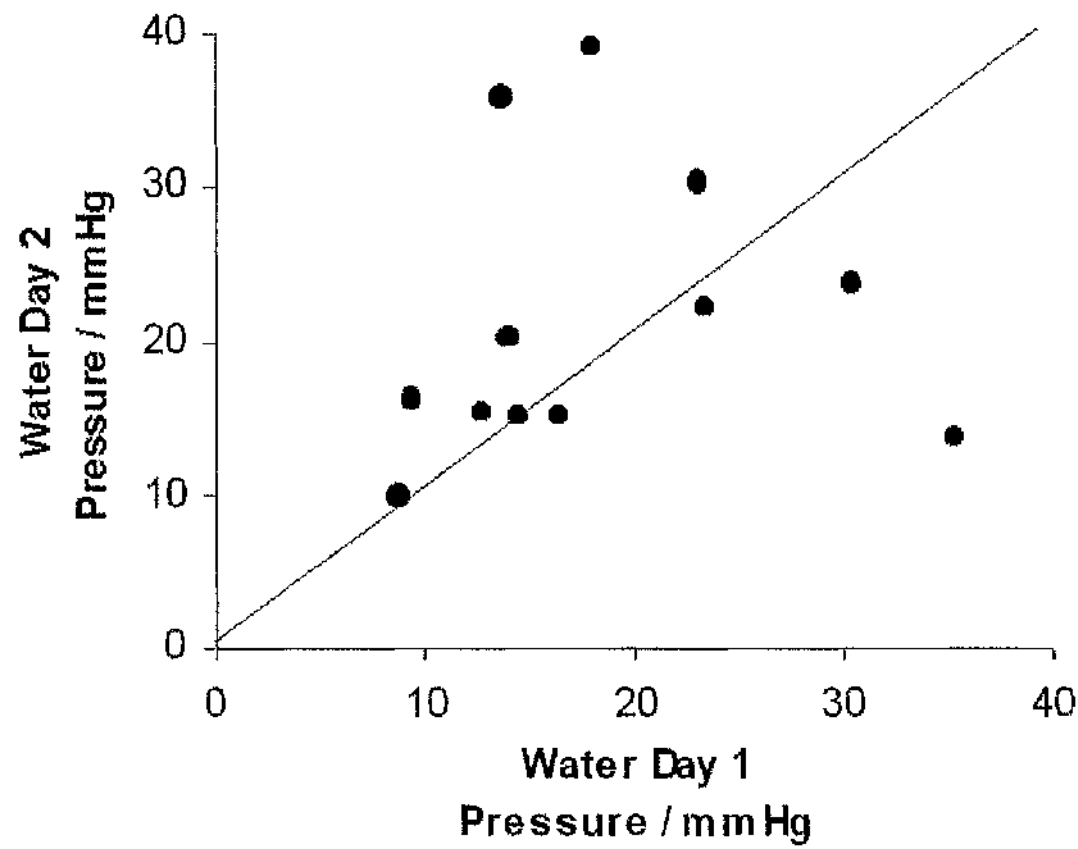
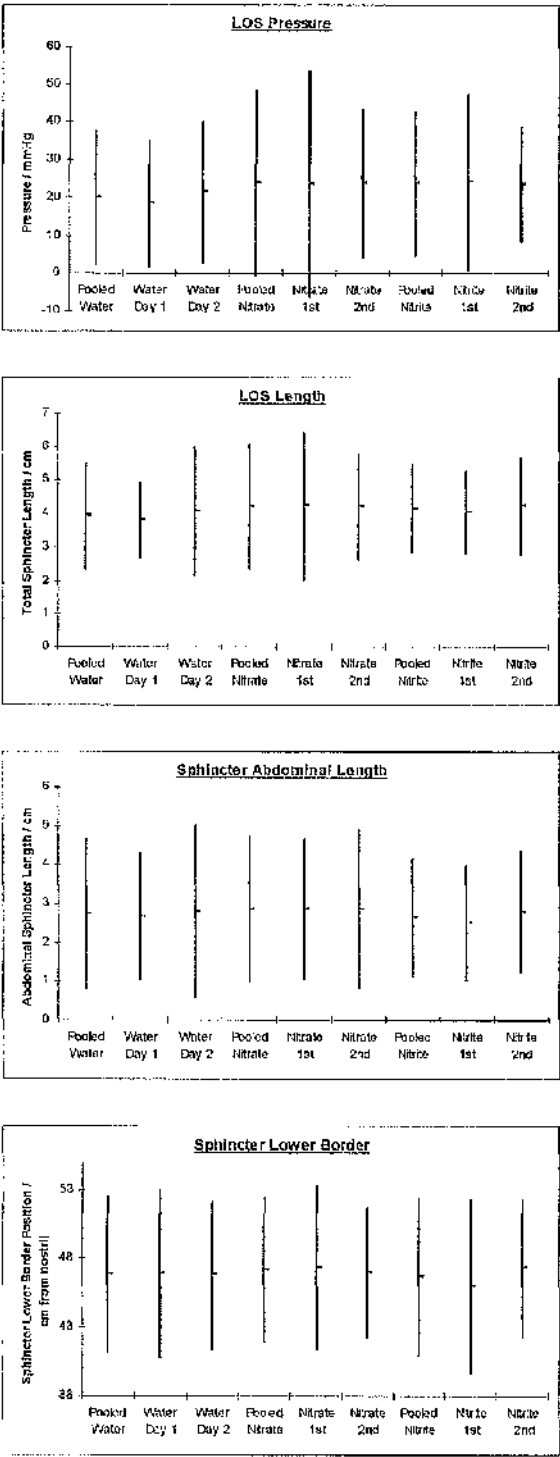


Figure 4.10: Charts to show 95% confidence intervals (Mean \pm 2SD) for the 4 measured sphincter parameters. These are subdivided into pooled data and subsets assessing the order of solution delivery.
 (Statistics for pooled data - Table 4.2, un-pooled data – Table 4.6)



***In vitro* assessment of nitric oxide gas production.**

Chapter 5

Chapter aims:

During creation and testing of the NO solution, with increasing concentrations of nitrite, a saturation point was reached. At this point NO came out of solution and manifested itself as gas bubbles lining the syringe. Although this appeared to be under control in the experimental equipment *in vitro*, we wanted to see if there was a possibility that this may still happen when the solution encountered acid and had left the tubing system.

A set of bench-top experiments was designed using a contained glass system, and modelling the experimental conditions of the meal study, when the amount of NO to be delivered would be greatest. NO gas volume produced was calculated by the differences in intra-container pressure caused on a vertical fluid column. The environment within the container was also modified to reduce oxygen levels, and increase inert volume, to assess the impact on NO production.

5.1 *In vitro* assessment of nitric oxide gas production

5.1.1 Aim:

To assess and quantify the possible contribution of NO gas formation following infusion of the test solutions into the stomach.

5.1.2 Background:

It is well characterised that TLOSRS occur as a result of gastric stretch and distension, primarily to the proximal stomach, and very likely the cardia itself. There is a theoretical risk that NO may come out of solution when it leaves the catheter and enters the stomach, forming a potentially significant volume of gas. 400 μ moles of NO gas infused over 1 hour could occupy 9.48m³ at 37°C and atmospheric pressure. With a patient sitting upright this gas would collect in the proximal stomach causing distension and potentially triggering TLOSRS.

5.1.3 Method:

In order to accurately measure gas volumes we needed to design a container to act as an 'artificial stomach'. The chamber would need to be of a set volume, so that gas production could be accurately assessed. A sealed glass chamber was used for this purpose. It was filled over three-quarters full with pH 0.56 HCl. We lowered one of our study manometry catheters into it. The opening through which this entered the chamber was sealed with an epoxy resin. Other joints were held with safety clips and sealed with a film of high vacuum grease (Dow Corning, USA).

Rising from the base and passing through the top of the sealed glass chamber was a hollow glass tube, approximately 8mm in internal diameter. It extended almost 80cm vertically from the chamber's base, and its upper end was open to the atmosphere. This would act as a manometer, from which we could calculate the pressure of the potential air/gas mixture in the sealed chamber. Prior to any experimentation this would mean that the manometer pressure was equilibrating with the air pressure in the head of the chamber. The complete apparatus was submerged into a water bath, sitting on a heater plate. This was warmed to 37°C to mimic physiological conditions. The sealed chamber had glass feet to elevate it from direct contact with the heater plate. The water bath contained a magnetic stirrer to maintain a uniform temperature – see Figures 5.1, 5.2 & 5.3.

Lowering the apparatus into the water bath caused a rise in temperature of both the gas and the acid in the sealed chamber. Although having a minor effect on the liquid, the gas expands, thus causing the level of the manometer to rise. Following a period of temperature equilibration, the manometer level was reduced, by withdrawing fluid back through one of the catheter lumens, until it was level with the main body of acid again.

The central lumen of the manometry catheter was sealed off with epoxy resin, as were the 4 proximal manometry channels. The same study infusions (Table 3.3) were then run through each of the 4 distal manometry channels at the standard study rates (99.9mls/hr/channel). The Nitrous Acid (NA) solution was identical to the NO generating solution apart from an absence of ascorbic acid from the 'gastric channels'. The Control solution contained ascorbic acid, but not nitrite. This set of solutions would be used in our main study (Chapter 6) and the solutions detailed in Table 6.1. Solutions

passed through identical lectro-caths and pressure transducers to mimic the study conditions.

5.1.4 Calculations:

The endpoint of the experiment would be when the manometer level had risen by a set vertical distance above its starting position. The volume of fluid needed to fill this part of the manometer column was measured. Recording the time needed for the infusion to produce this rise was documented for each solution. Prior to any experiments, the total volume of the sealed chamber was measured, as were the initial volume of hydrochloric acid and air (constant for all experiments). Simply by subtracting the amount of fluid infused from that in the manometer column would show how much had stayed in the chamber. If the NA (NO solution minus Ascorbic Acid) or NO solutions needed to infuse less volume than the control, then the difference would be the volume occupied by NO gas formation.

This volume of gas would be at atmospheric pressure plus the pressure from the manometer column. The volume at 37°C could then be recalculated at atmospheric pressure alone. There is always a little 'dead-space' within the lectro-caths and length of the manometry catheter. For this reason, the cumulative dead-space was measured for all 4 channels. This could be subtracted from the total volume infused to give the actual volume of active test solution reaching the sealed chamber. Depending on the duration of the infusion, the gas volume could be extrapolated to suggest a potential volume of generation over the total hour infusion. This calculation would assume a linear production of gas over the hour infusion.

5.1.5 Solutions:

The apparatus was first tested with distilled water followed by the study Control solution (Table 5.1). These baseline recordings would hopefully confirm that the Control solution itself did not produce any gas. Following this, the experiment was repeated with NA and then NO generating solutions in the syringes (Table 5.2). All test solutions including the distilled water in the manometry dead-space were deoxygenated by pre-gassing them with Argon.

5.1.6 Results:

Total volume of artificial stomach = 540mls

Initial ratio of 476mls acid & 64mls of air

Volume of column of fluid caused by infusion = 23.5mls

Endpoint - duration of infusion to achieve manometer height of 0.7m

Distilled water / Control = 5 minutes (33.3mls infused)

NA = 4 minutes 45 seconds (31.7mls infused)

NO = 4 minutes 30 seconds (30mls infused)

Pressure calculations

Pre-infusion pressure in stomach chamber = atmospheric (100Pa)

Post-infusion pressure = atmospheric + pressure from fluid column

$$= 100 + (\rho \times g \times h)$$

$$= 100 + (\text{solution density} \times \text{acceleration due to gravity} \times \text{column height})$$

[density of solution is 1.00 to 3 significant figures, $g=9.81\text{ms}^{-2}$]

$$= 100 + (1 \times 9.81 \times 0.7)$$

$$= 106.86\text{Pa}$$

Gas volume

Volume of gas produced at 106.86Pa

= difference between infusion volumes compared to control

Volume of NO gas from NO generating solution

$$= 33.3 - 30\text{mls at } 106.86\text{Pa}$$

$$= 3.3\text{mls at } 106.86\text{Pa}$$

$$= 3.53\text{mls at atmospheric pressure (sea level) and } 310\text{K (} 37^{\circ}\text{C)}$$

Volume of NO gas from NA solution

$$= 33.3 - 31.7\text{mls at } 106.86\text{Pa}$$

$$= 1.6\text{mls at } 106.86\text{Pa}$$

= 1.7mls at atmospheric pressure (sea level) and 310K (37°C)

Predicted NO gas generation

Dead space of tubing prior to reaching glass chamber is 4 x 2.4mls (supply to each of the distal manometry ports).

Therefore, the volumes of active solution infused are

Control: $33.3 - (4 \times 2.4) = 23.7\text{mls}$

NA: $31.7 - (4 \times 2.4) = 22.1\text{mls}$

NO: $30 - (4 \times 2.4) = 20.4\text{mls}$

Assuming linear NO release over one hour (400mls infused) this could potentially generate

= $3.53\text{mls} \times (400/23.7)$

= **59.5mls** of NO gas over 1 hour from NO generating solution at atmospheric pressure (sea level) and 310K (37°C)

Potential NO from NA here

= $1.7\text{mls} \times (400/22.1)$

= **30.8mls** of NO gas over 1 hour from NA solution at atmospheric pressure (sea level) and 310K (37°C)

5.1.7 Discussion:

We have devised a system that allows the infusion solution to be captured in a sealed chamber. In doing so, and by use of a manometer, we have been able to assess the amount of NO gas released by each of our study solutions over a short duration. Performing linear extrapolation, we have been able to estimate the potential volume of NO gas that may be produced over the full hour infusion.

5.1.8 Conclusion:

This experiment confirms the generation of NO gas from our study solutions. However, these are slightly confusing results, in that the NA solution produces almost 50% of the volume of gas as the NO solution. In order to investigate our concerns over the relatively low volumes of NO gas produced we decided to devise a further set of experiments. As stated, at 37°C, if all of the NO were to come out of solution and assume gaseous form, the total amount (400 μ moles) could occupy 9.48 m³ at sea level.

We calculated that approximately 60mls of NO gas would be produced, which equates to 6x10⁻⁶% of that expected. Also of concern was the amount of NO gas produced from the saturated NO solution, relative to the volume of NO gas produced by the NA solution (NO approximately 1% by concentration). When the chemical reaction was examined, we were able to postulate several possible explanations.

With NA, the low NO concentration means that the NO is more likely to remain as NO in solution. However, with the higher NO concentration in the NO generating solution, it has potential to react rapidly with the O₂ in the HCl and head of air in the chamber. This will form nitrite, which the Vitamin C will convert back to NO. However, if this recycling depletes the Vitamin C, which will happen very quickly, then the NO will stay as nitrite, thus reducing the amount of NO gas formed.

Secondly, although the NO concentration is very high coming out of the catheter for the NO generating solution, once it enters the chamber it will be rapidly diluted by the large quantity of acid. This may, in theory, lower its concentration closer to that of the NA solution, reducing the tendency of the NO to preferentially leave solution. It is relatively important to know the

answer to this, as it will reassure us as to whether the NO effects seen in our study, were pharmacological, physiological, physical, or a combination.

5.2 *In vitro* assessment of dissolved oxygen in the recycling and formation of NO and nitrite.

5.2.1 Aim:

To see the impact of deoxygenating the artificial stomach on NO gas formation.

5.2.2 Method:

Using the same apparatus as before, the NO generating experiment was repeated with slight modifications. All solutions were prepared with argon deoxygenation. For extra thoroughness, all solutions had a head of argon placed above them before replacing the lid. This was achieved by suspending the gassing stone above the level of the solution for approximately 1 minute. Also, the pH 0.56 HCl in the artificial stomach (476mls) was deoxygenated with argon gassing. Also, an argon head was delivered to replace the air in the chamber, before placing the airtight cap.

The same starting volumes of acid and gas in the chamber were used. Again, it was lowered into a water bath at 37°C, and after a period of temperature equilibration, the NO generating infusion was commenced. The previous control experiments were used as the control comparison for this run. Gas volume calculation was performed as previously described.

5.2.3 Results:

Endpoint - duration of infusion to achieve manometer height of 0.7m

NO (fully deoxygenated) = 4 minutes 30 seconds (30mls infusion)

Control (non-deoxygenated) = 5 minutes (33.3mls infused)

NO gas generated in chamber

= 33.3mls – 30mls at 106.86Pa

= 3.3mls at 106.86Pa

= **3.53mls** at atmospheric pressure (sea level) and 310K (37°C)

5.2.4 Discussion:

As well as continuing to deoxygenate all test solutions prior to mixing and the dead-space distilled water, we have also removed the oxygen from the contents of the artificial stomach by pre-gassing with argon. We have used the same volume of acid in the artificial stomach as in the previous experiment. When the concentrated NO solution is infused into the submerged, sealed chamber, there is no change in the volume of NO gas produced.

5.2.5 Conclusion:

Deoxygenation of the HCl in the artificial stomach does not appear to have an impact on the production of NO gas from the concentrated NO solution. However, dilution of the NO solution from the catheter tip into the large volume of the chamber's acid must be taken into account, and may explain our results. The next experiment was designed to address this.

5.3 Assessment of concentrated NO solution dilution on its ability to produce NO gas in a deoxygenated environment

5.3.1 Aim:

To assess if NO gas production, in a deoxygenated environment, is more prolific from a concentrated NO solution.

5.3.2 Method:

Once again the same artificial stomach was used for this experiment. All solutions, including the acid in the artificial stomach were deoxygenated. Heads of argon gas were placed in the chamber and also into the solutions' containers before they were mixed. A smaller volume of acid was placed into the artificial stomach (78mls compared to 476mls previously) – see Figure 5.4. This was the lowest amount that would cover the lower end of the manometer column, and also not cause major issues with chamber buoyancy. Due to a lower volume of acid in the artificial stomach and a greater amount of gas, this meant that the chamber had to be forcibly held by clamps to the bottom of the water bath for the duration of the experiment.

Distilled water was used as a control run. All 4 syringe drivers were set at 99.9mls/hr/channel and delivered their infusion into the chamber until the manometer column had reached the target vertical limit as set before. The experiment was then repeated with the NO generating solution, and deoxygenated HCl in the chamber. As the fluid level in the chamber at the start of the experiment was lower than before, this meant that the change in height of the manometer fluid column would be greater than before. This height was measured and also the volume of fluid needed to achieve this.

5.3.3 Results:

Total volume of artificial stomach = 540mls

Initial ratio of 78mls acid and 462mls of Argon

Height of manometer column at end of infusion = 76.5cm

Volume of fluid to fill the manometer column = 27.5mls

Endpoint - duration of infusion to achieve manometer height of 0.765m

Distilled water control = 9 minutes 39 seconds (64.3mls infused)

NO generating solution = 9 minutes (60mls infused)

Volume of NO gas produced = 4.3mls after 9 minutes

Pressure acting on gas in chamber at 37°C:

= Atmospheric + column of manometer fluid

$$= 100 + (\rho \times g \times h)$$

$$= 100 + (1 \times 9.81 \times 0.765)$$

$$= 107.7\text{Pa}$$

Volume of NO gas at atmospheric pressure and 37°C

$$= 4.66\text{mls}$$

Volume of NO solution infused:

$$= 60 - (4 \times 2.4)$$

$$= 50.4\text{mls}$$

Potential volume of NO gas generation, assuming linear production

$$= 4.66 \times (400/50.4)$$

$$= \underline{37\text{mls}}$$

Calculated concentration of NO in the artificial stomach after dilution

Original experiment with 'full' stomach

$$= 1.1\text{mmol/l} \times 30\text{mls}/(30\text{mls} + 476\text{mls})$$

$$= \underline{0.065\text{mmol/l}}$$

Repeat experiment with 'empty' stomach

$$= 1.1\text{mmol/l} \times 60\text{mls}/(60\text{mls} + 78\text{mls})$$

$$= \underline{0.48\text{ mmol/l}}$$

Ratio of end NO concentrations in chamber for each experiment

$$= 0.48\text{mmol/l} / 0.065\text{mmol/l}$$

$$= 7.36 \text{ fold difference}$$

5.3.4 Discussion:

We have adapted the artificial stomach system from our original experiment. The oxygen has been completely removed from the system to the best of our ability, and also the starting volume of acid in the chamber has been reduced in order to minimise the dilution of the NO solution entering the chamber. Despite these measures there does not appear to be any dramatic increase in the volume of NO gas produced. Also, the predicted volume of NO gas from this last experiment is actually less than that of the original run. This implies that the release of NO from solution to form gas is not linear.

There is a low O_2 tension within both gastric juice and the stomach. The amount of vitamin C present is critical to the amount of NO produced. There does, however, not appear a great amount of NO gas generated, even under theoretically ideal conditions.

5.3.5 Conclusions:

Due to the variable availability of vitamin C at the cardia, and from our results with the artificial stomach, it is highly unlikely that a large volume or even small 'blasts' of NO gas would be formed. Certainly, there would not be a large enough volume to cause a physical distension of the proximal stomach, triggering TLOSRS.

5.4 Assessment of gas volume head-space on the ability of NO gas production from a concentrated NO solution.

5.4.1 Aim:

To assess if the pre-existing volume of gas in the artificial stomach's head space plays a role on the amount of NO gas generated from the concentrated NO solution.

5.4.2 Method:

The same artificial stomach set up was used as before. The initial volume of acid in the stomach was again 78mls prior to the start of the infusion. This had been pre-gassed with argon to remove the oxygen. A head of argon gas was also added to the chamber before closure. Added to the glass chamber was a known volume amount of glass marbles (Figures 5.5 & 5.6). This inert occupation of space would allow for a reduction in the volume of gas in the chamber and thereby reduce the gas/liquid volume ratio. The potential concentration of NO in solution will be dictated by the volume of acid into which the concentrated NO solution is infused – see Table 5.4.

The volume occupied by the marbles was calculated by their displacement of water in a measuring cylinder. Two different volumes of marbles were used, so that, including the marble-free run already performed, we would have 3 points from which a relationship or trend could be observed. Once lowered into the water bath and temperature equilibration had taken place, the NO generating solution (Table 3.3) was infused at the standard rate (99.9mls/hr/channel). The initial volume of gas in the chamber was calculated by subtraction of the marbles volume from 462mls. Volume of NO

generated was calculated as before, using the time to reach a set rise in the manometer height as the endpoint.

5.4.3 Results: (summarised in Table 5.3)

Run 1 with control:

Volume of marbles added to chamber = **105mls**

Volume of acid in chamber = 78mls

Volume of Argon in chamber = 357mls

Time taken for infusion to reach end-point:

NO generating solution = 7 minutes 51 seconds

Distilled water = 8 minutes 5 seconds

Infusion volumes:

NO generating solution = 52.3mls

Distilled water = 53.8mls

Volume of NO gas generated = 1.5mls

Volume of fluid to fill manometer column to end-point = 26.5mls

Vertical height of manometer to reach end-point = 81cm

Pressure from column = $(\rho \times g \times h)$

= $1 \times 9.8 \times 0.81$

= 7.94 Pa

Volume of gas at 1 atmosphere (100 Pa)

= 1.6mls

Potential volume of NO gas from total infusion

= $(400/52.3) \times 1.6$

= **15mls**

Run 2 with control:

Volume of marbles added to chamber = **205mls**

Volume of acid in chamber = 78mls

Volume of Argon in chamber = 257mls

Time taken for infusion to reach end-point:

NO generating solution = 6 minutes 15 seconds

Distilled water = 6 minutes 39 seconds

Infusion volumes:

NO generating solution = 41.6mls

Distilled water = 44.3mls

Volume of gas generated = 2.7mls

Volume of fluid to fill manometer column to end-point = 26.5mls

Vertical height of manometer to reach end-point = 81cm

Pressure from column = $(\rho \times g \times h)$

$$= 1 \times 9.8 \times 0.81$$

$$= 7.94 \text{ Pa}$$

Volume of gas at 1 atmosphere (100 Pa)

$$= 2.9 \text{ mls}$$

Potential volume of NO gas from total infusion

$$= (400/32) \times 2.9$$

$$= \underline{\underline{36.25\text{mls}}}$$

Volume of NO generating solution entering chamber excluding dead-space:

105mls of marbles = 42.7mls

205ml of marbles = 32mls

5.4.4 Discussion:

This set of studies reveals a little more about the manner in which NO comes out of solution. It gives some insight into the effects of the liquid:gas ratio on NO gas release. It also shows the effect of different [NO], caused by solution dilution in the chamber, on gas production in a similar environment. There are several factors that need to be taken into account to interpret this data. We know that the release of NO gas from solution is unlikely to be linear. Our endpoints are when a similar pressure column has been attained.

As there are different volumes of the same NO study solution being infused, and the volume of acid in the chamber stays the same, the potential concentration of NO in the chamber liquid is different for each. In the run with no marbles the calculated concentration of NO is 0.40mmol/l. For the 105mls of marbles run the NO concentration is calculated at 0.36mmol/l, and the 205mls of marbles run at 0.29mmol/l.

Therefore, the amount of gas produced in each of these runs is going to be multifactorial ie: based upon the concentration of NO in the chamber and also the ratio of volume of liquid and gas existing in the chamber. This is clear to us, as the amount of gas potentially produced from the marbleless chamber is 37mls. This is similar to the 205mls of marbles run (36.3mls), but higher than the 105mls of marbles run (15mls). This is despite the concentration of NO in the chamber liquid decreasing in concentration with each experiment. There are also possibly some errors which have not been taken into account.

As our gas to liquid ratio is probably still a little high even for the 205mls of marbles run (78mls of acid and 257mls of Argon), this would imply that in a post-meal stomach, the amount of NO gas generated may be even

higher than we have predicted. Even so, the level is unlikely to be anything like that required to cause marked distension of the proximal stomach triggering TLOSRS.

5.4.5 Conclusions:

The 3 points do not form a linear relationship as initially one might expect. This indicates several points. Firstly, NO is not behaving as an ideal gas. This is mainly as there is a gas liquid interface and Boyle's law ($\text{Pressure} \times \text{Volume} = \text{Constant}$) does not therefore hold. Also, the change in surface area of the acid and NO solution may play a role in this equilibrium. Finally, the ratio of acid to chamber gas also appears to play a key role in the movement of NO between the gas and liquid phases.

Table 5.1: List of the constituents of the control solution for the 'meal' study.

Gastric channels	Oesophageal channels
Control study day	
Thiocyanate – 1mM	Thiocyanate – 1mM
Hydrochloric acid – pH 1.0	
EDTA – 1mM	
Ascorbic Acid – 5mM	

Key: 'Gastric' channels refers to the distal 2 manometry channels and 'Oesophageal' channels to the 2 channels proximal to this.

Table 5.2: Summary of solution concentrations and catheter tip concentrations. NA – nitrous acid, NO – nitric oxide.

Solution	[Nitrite] in syringe / mmol	[NO] at catheter tip / mmol
NA	1.6	0.012
NO	1.6	1.1

Table 5.3: Summary of NO production with altered starting chamber Argon volume and calculated end [NO] in the chamber.

Volume occupied by marbles (ml)	Vol of NO solution added (ml)	[NO] in stomach (mmol/l)	Vol of NO gas produced (ml)	Pressure from column (Pa)	Calculated volume of NO over 1 hour (ml)
0	50.4	0.40	4.66	107.7	37.0
105	42.7	0.36	1.6	107.9	15.0
205	32	0.29	2.9	107.9	36.3

Table 5.4: Illustration of how volume of infused acid and container acid volume will affect final NO solution concentration.

$$\text{Final [NO] in chamber} = \text{NO concentration at catheter tip (1.1mM)} \times \frac{\text{Volume of NO solution added}}{\text{Starting volume of acid in chamber} + \text{Dead space volume in tubing} + \text{NO solution volume infused}}$$

Figure 5.1: Cap of stomach chamber with manometry tube secured.

Key: **A:** Epoxy resin seals, **B:** Manometry catheter, **C:** Manometer column for stomach, **D:** Glass cap for stomach, **E:** Top aperture for manometer extension.

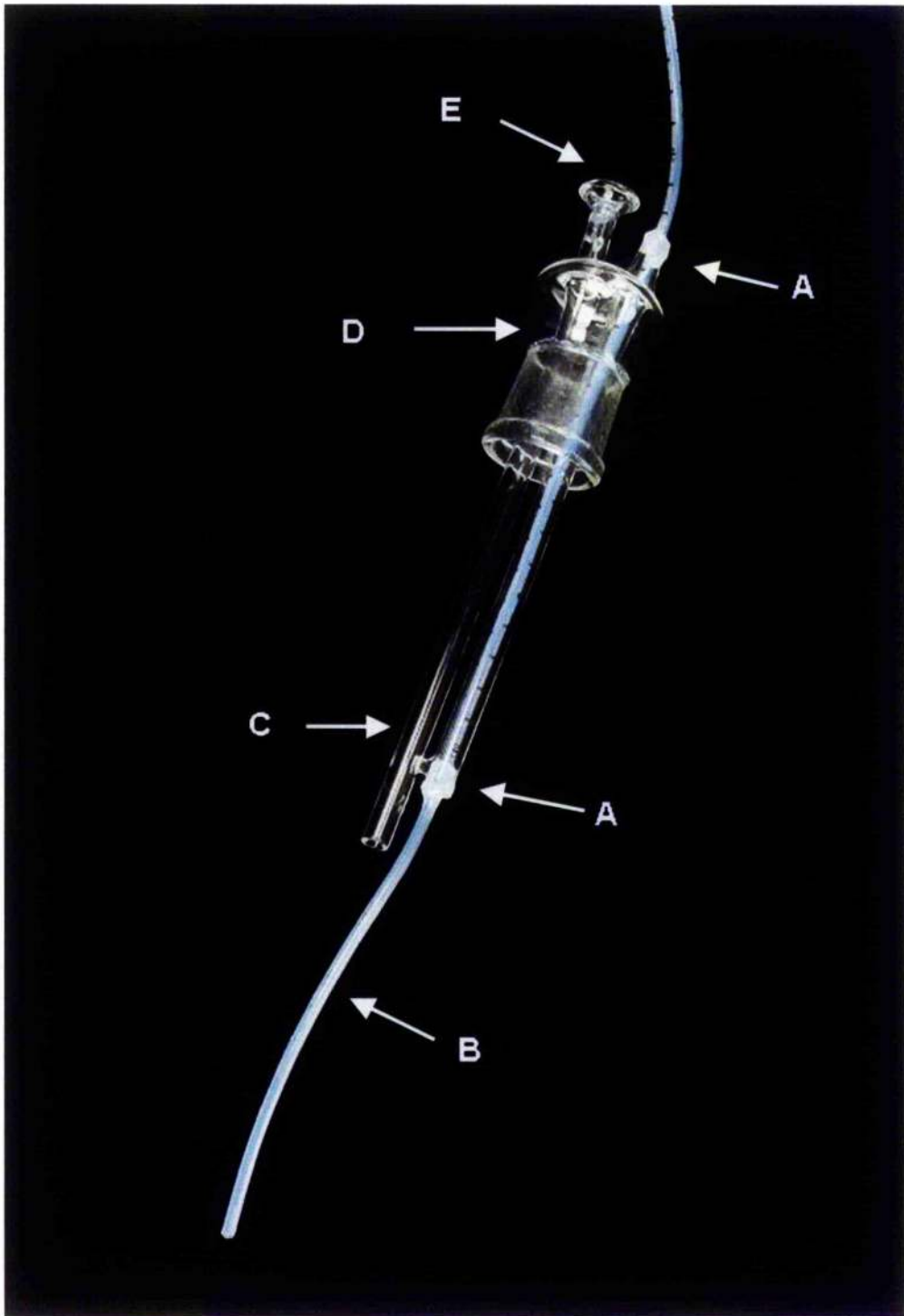


Figure 5.2: Artificial stomach with large acid volume.

Key: **A:** Oxygenated hydrochloric acid (pH 0.56) (476mls), **B:** Air in head space (64mls), **C:** Securing metal clip, **D:** Manometry column, **E:** Joint sealed with high vacuum grease, **F:** Glass feet



Figure 5.3: Submerged artificial stomach.

Key: A: Securing plastic clip, B: Water at 37°C, C: Thermometer, D: Heater plate, E: Magnetic stirrer

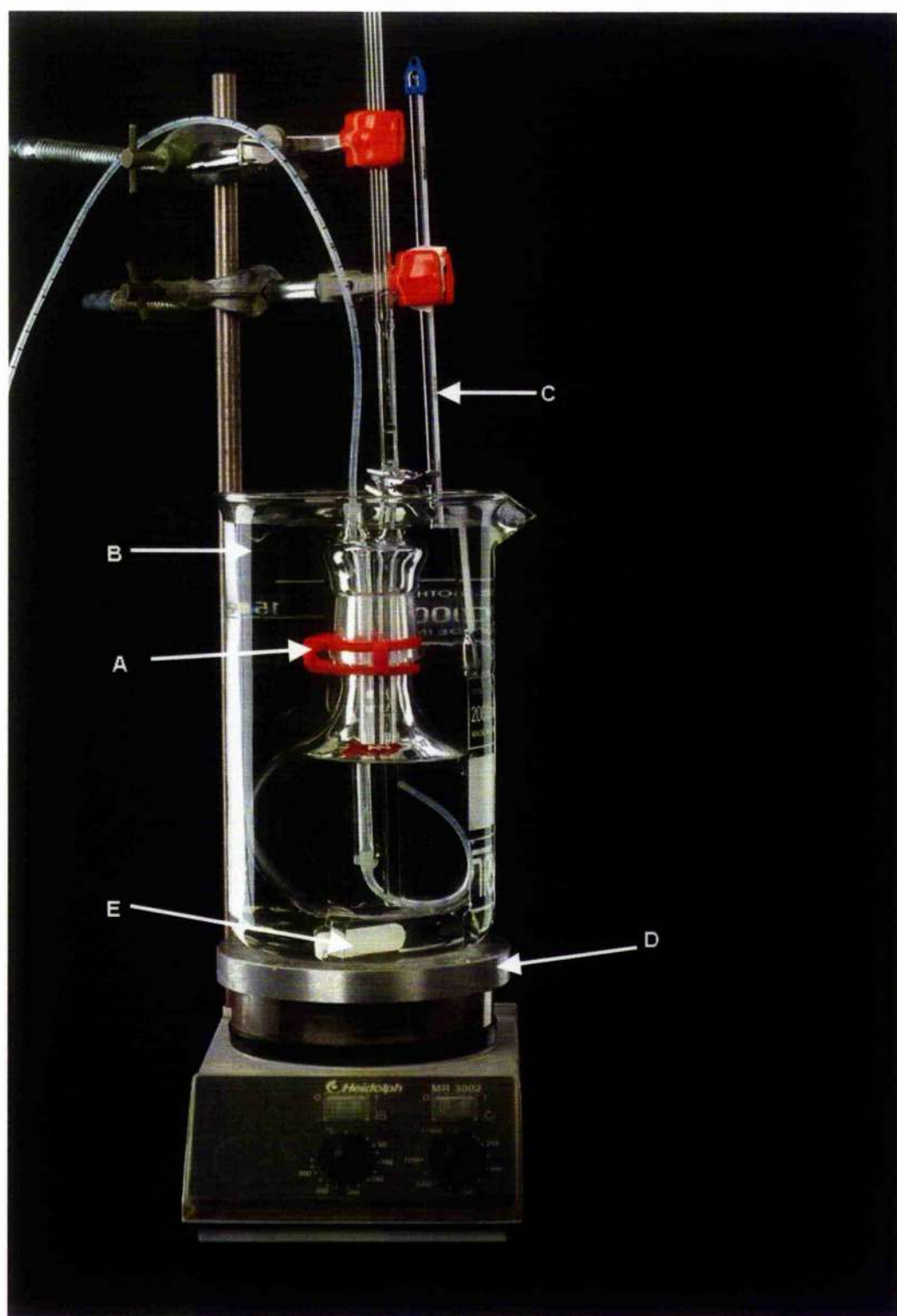


Figure 5.4: Artificial stomach with small acid volume.

Key: **A:** De-oxygenated hydrochloric acid (pH 0.56) (78mls), **B:** Argon in head space (462mls)



Figure 5.5: Artificial stomach with inert occupation by small volume of marbles.

Key: **A:** De-oxygenated hydrochloric acid (pH 0.56) (78mls), **B:** Argon in head-space (357mls), **C:** Marbles in chamber (105mls).

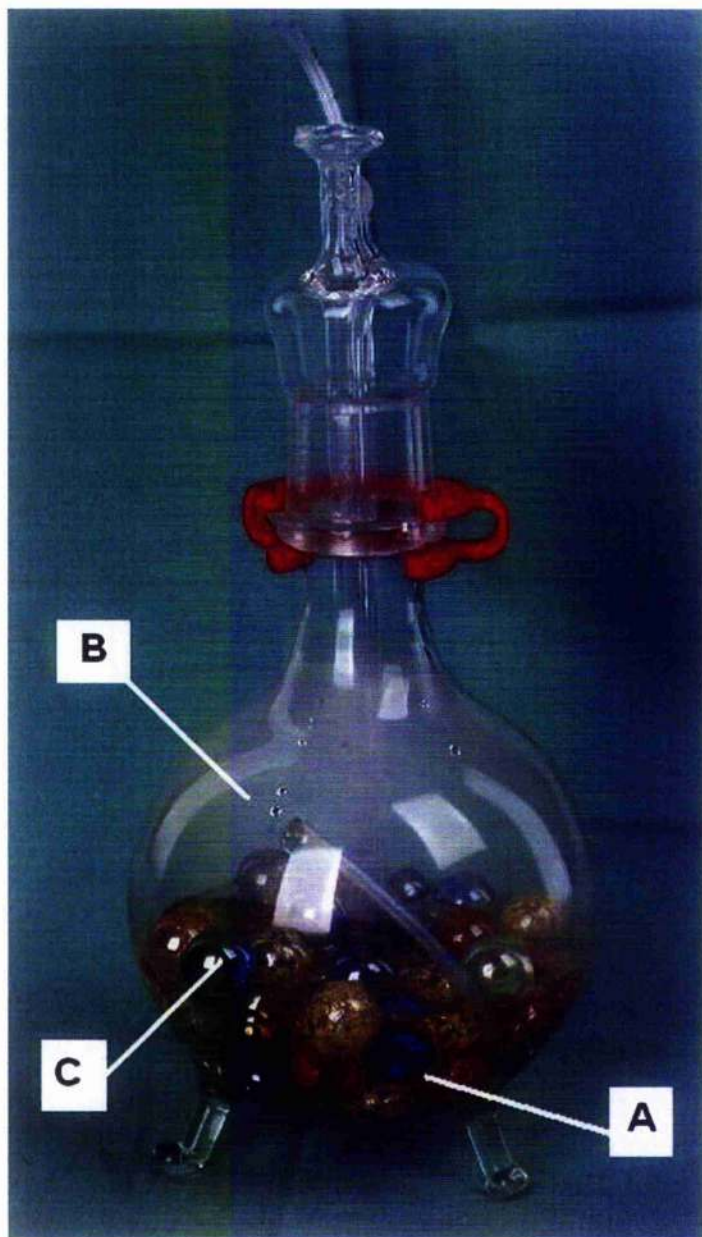
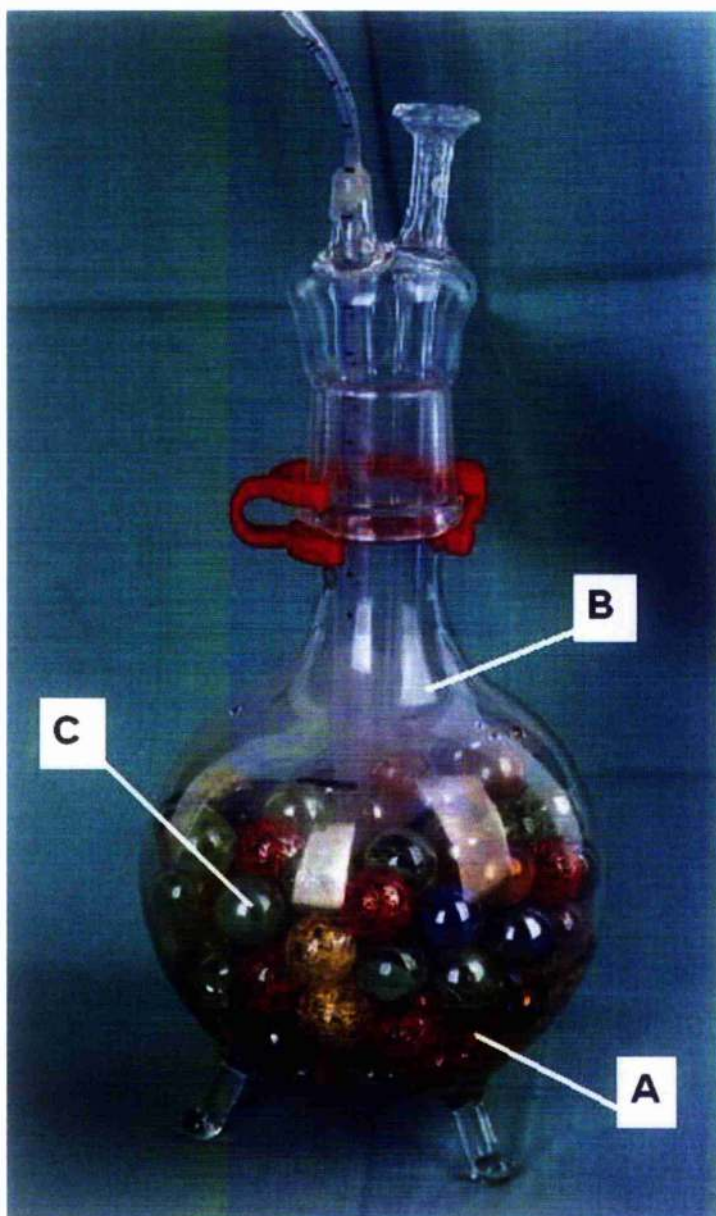


Figure 5.6: Artificial stomach with inert occupation by large volume of marbles.

Key: **A:** De-oxygenated hydrochloric acid (pH 0.56) (78mls), **B:** Argon in head-space (257mls), **C:** Marbles in chamber (205mls).



**The influence of luminal nitric oxide and
nitrous acid on human LOS pressure and
oesophageal pH, in the post-prandial state.**

Chapter 6

Chapter aims:

This chapter examines the physiological effects of the nitric oxide and nitrous acid solutions delivered to the cardia region of the human upper gastrointestinal tract. As there was no effect seen in the fasting study, this study is carried out following a meal, when the upper gastrointestinal tract is under more direct stress and both TLOSRS and reflux are known to be more common. Higher doses of the luminal chemicals are used to model the increased levels present in saliva, as would be expected following a meal. The primary end-point of the study will be to look at the effects of the solutions on LOS pressure. Other secondary end-points, such as post-prandial TLOSRS rates and intra-oesophageal pH will also be addressed.

6.1 Background:

Gastro-oesophageal reflux disease is caused by excessive acid exposure of the oesophagus. Luminal acid is neutralised by secreted mucosal bicarbonate and the delivery of saliva. This is carried into the stomach by both a wave of peristalsis, and gravity when in the upright posture. TLOSRS are the major mechanism of reflux events in both healthy subjects and also those with reflux disease. Nitric oxide antagonists have been shown to inhibit the triggering of TLOSRS by gastric distension. The physiological effects of lumenally generated NO, in the proximal gastrointestinal tract of humans, is not known.

Human saliva has a high concentration of nitrite, which is derived from the enterosalivary recirculation of nitrate (232). Approximately 25% of the nitrate absorbed from the small intestine, and produced endogenously, is taken up by the salivary glands and secreted into the mouth (233). 10-90% of the nitrate in saliva is reduced to nitrite by bacteria on the dorsum of the tongue (234).

When saliva is swallowed and encounters the acidic pH of the cardia, the nitrite within it is immediately converted to varying proportions of NA and NO (235). If ascorbic acid or other reducing agents are present in adequate amounts in the acidic gastric juice then all the nitrite is converted to NO in a 1:1 molar ratio. In the absence of reducing agents the nitrite is converted to nitrous acid and only 1% to NO (236). The NA will also react with available thiols to form nitrosothiols (196).

Nitric oxide plays an important role in the physiological regulation of gastro-oesophageal motility. Neuronally released NO and NO donors such as nitrosothiols relax the body of the oesophagus, the LOS (196) and also the

body of the stomach (141). This is mediated by NO increasing activity of cGMP (179). Increased local availability of NO could explain several of the pathophysiological features of GORD, including transient relaxations of the muscularis externa in the LOS, reduced sphincter tone and impaired oesophageal clearance.

The fact that substantial amounts of NO and nitrosothiol chemicals are generated when saliva meets gastric acid raises the possibility that this luminal chemistry might affect gastro-oesophageal motility and potentially contribute to the pathogenesis of reflux disease. Such an effect would depend upon the ability of these chemicals generated within the lumen to exert an effect on the musculature within the adjacent mucosa.

6.2 Aim:

The aim of this study was to determine whether luminally administered NO and/or NA could influence the generation of TLOSRS following a meal. Data on IGP, oesophageal acid exposure and peristalsis would also be collected during the study.

6.3 Study population:

15 healthy volunteers were recruited for this study. All were found to be *H. pylori* negative by urea breath test. We defined a healthy volunteer as someone with no upper gastrointestinal symptoms and no past history of upper gastrointestinal surgery. None were taking any form of acid suppression medication or antacids. We examined 8 males, average age 31 years (range 23-43) and 7 females, average age 41 years (range 31-58). Body mass index was slightly higher in the females, mean 26.7 (range 21.2-

30.5) and 25 for the males (range 19.3-29.8). Smokers were allowed to participate in the study but did not smoke on the day of their investigation.

6.4 Method:

6.4.1 Overall protocol:

Studies of the effect of luminal NO and NA on gastro-oesophageal function were performed after eating, as this is the time when the salivary delivery of these chemicals is maximal. It is also the time when TLOSRS characteristically occur with subsequent acid reflux (237). Each patient had SMPT manometry of their LOS performed (49), before and after a meal to identify the HPZ. Following the meal, test solutions were infused for one hour into the cardia region of the stomach. Throughout the infusions static manometry recordings of both the HPZ and oesophageal body was performed, as well as pH recordings of the oesophagus. Post-infusion SMPT manometry of the HPZ was also performed. Each subject attended for 3 study days, receiving on each day in a double-blind randomised order, one of the following infusions: Hydrochloric acid (control), hydrochloric acid plus nitrite (nitrous acid), or hydrochloric acid plus nitrite plus ascorbic acid (NO generating solution) - (Table 6.1 & Figure 6.1).

6.4.2a Details of protocol:

All participants were instructed regarding measures to reduce their endogenous salivary nitrite content over the 48 hours prior to each test. This included avoiding high-nitrate containing foods i.e.: salads, green leafy vegetables, potatoes, beetroot and gargling with 15mls of chlorhexidine gluconate mouthwash 0.12% w/v (Chlorohex 1200, Colgate) twice a day, to reduce bacterial reduction of salivary nitrate to nitrite (163).

Subjects attended fasted on the day of each study. While seated upright the manometry/pH probe assembly was introduced into the nasal airway and swallowed until at least 65cm had been passed. Even without active manometry perfusion, but with static columns of water in the manometry catheter, passage into the stomach could be confirmed manometrically by both deep inspiration and also by the significant drop in pH as recorded by the pH probes.

The patient was then given 15 minutes to get used to the sensation of the apparatus before recording began. At this stage, no solutions were running through the manometry catheter. A 1cm-interval station pull-through, at 1 minute time intervals, was performed to locate the pH-step-up point, which in the fasting state, is just distal to the SCJ (23).

The apparatus was then passed back into the stomach and all 8 pressure channels were perfused with de-oxygenated distilled water at the flow-rates described in the manometry section (6.4.2b). With the subject reclined at 20° to the horizontal a SMPT was performed (Catheter Puller, Medtronic Functional Diagnostics A/S, Denmark), using a withdrawal rate of 1mm/second. This was done during normal respiration and outwith any swallowing events. This gave a fasting baseline profile of the LOS, from which we could calculate a) the total length of the high pressure zone and its position relative to the nares, b) the RIP and therefore the length of the abdominal component of the sphincter c) the MEEP relative to the gastric baseline pressure and d) the position of the pH step-up/SCJ relative to the HPZ.

The distal four pressure channels only were used to assess these parameters, and all channels were withdrawn from the stomach into the

oesophageal body. Proximal oesophageal channels were also perfused with distilled water at the same time to detect peristalsis, as all HPZ analyses needed to be performed outwith swallowing events. The syringes used to supply the distilled H₂O were different to the ones used for the test solutions in the later experiments.

Following the SMPT the manometry perfusion was stopped and the apparatus passed back into the stomach. The patient sat up and was given a standard battered fish and chip meal with a glass of water to drink. Acid buffering within the stomach could be observed with the pH probes in situ. One to two minutes after ingestion of the meal the patient lay flat and the SMPT was repeated with deoxygenated distilled water running through all channels. This allowed us to see the degree of shortening of the sphincter caused by the meal and also any coincidental changes related to pressure, again using the fasting gastric baseline pressure as a reference value.

The patient then sat upright and the apparatus was passed back into the stomach. It was withdrawn to the point where the most distal channel was recording gastric pressure, and according to the pressure pull-through profile, the channel proximal to this was recording pressure in the lower part of the sphincter. The manometry and pH assembly was then taped to the nose for the one-hour recording period – see Figure 6.2.

With the assembly positioned, perfusion of the test solutions was commenced through the lower 4 manometry channels and distilled water through the oesophageal channels. Each of the syringes contained 50mls of solution running at 100ml/hour. Therefore, each syringe needed to be changed halfway through the recording process, creating a brief hiatus for data acquisition. It is important to note that we were using the perfusion

system for a dual purpose, to deliver test solutions and to monitor pressure. Subjects were not allowed to speak or drink during the recording period to avoid unnecessary activation of the swallow sensor.

After the 60 minute perfusion period the manometry assembly was reinserted into the stomach, the subject lay flat, and a final SMPT performed, again using deoxygenated distilled water as the perfusate through all 8 manometry channels. This pull-through was performed several minutes after the infusion had stopped and was, therefore, no longer bathing the cardia region.

6.4.2b Manometry:

A Muiscentific water perfused manometry pump was used (PIP-4-8, Muiscentific, Canada). Recordings were transferred through pressure transducers (Mediplus, 203216, High Wycombe, UK) via a 12 channel Polygraf (Medtronic Functional Diagnostics A/S, Denmark) to a personal computer and assessed using the Polygram Net software package (Version 4.01.525.45, Medtronic A/S, Denmark). A 3.8 millimetre eight lumen manometry catheter was used (2282, Mediplus, UK). This catheter was made from medical-grade PVC. With 5 centimetres of distal dead-space, moving proximally, the port intervals were 1cm, 1cm, 1cm, 2cm, 5cm, 5cm and 5cm. The 4 most proximal catheter ports were perfused with deoxygenated distilled water from the manometry chamber at a pressure of 15Kpa, giving a flow rate of 0.45mls/minute/channel.

The 4 lower catheter channels were supplied by individual syringe pumps (Perfusor, Braun, Berlin, Germany) connected to the efferent pressure transducer port by 1mm internal-diameter PVC tubing (1155.10, Vygon, Cirencester, UK). Letrocaths were used in place of standard capillary tubing,

as the resistance generated was too great for the syringe drivers to overcome at an acceptable flow rate. As larger calibre tubing was in use, all 4 syringe pumps were placed below the level of the manometry catheter ports to prevent siphoning. The syringes delivered solutions at their maximum flow-rate of 1.67mls/min for each individual channel. The solution delivered by each pump is described below. All manometry channels were calibrated, whilst running at their experimental flow-rates, in a 2-point fashion at zero and 680mmH₂O (equivalent to 50mmHg).

6.4.2c pH:

A 2-channel antimony crystal paediatric pH probe (26270, Mediplus, UK) was used to assess pH and reflux events during this study. The probe was 1.5mm in diameter, with an external reference electrode, and the 2 sensors positioned 7cm apart. The 2 sensors and reference electrode underwent a 2-point calibration in Phosphate-free buffer at pH 1.07 and pH 7.01 (Medtronic A/S, Denmark). The pH probe was attached to the manometry catheter with thin strips of adhesive tape (Mefix, Tendra, Sweden) so that the lower recording sensor was 6 cm proximal to the distal manometry port - see Figure 6.2.

6.4.2d Swallow sensor:

A small balloon swallow sensor was taped over the cricoid cartilage to assess co-ordination of oesophageal peristalsis (RespSponseTM Transducer, Medtronic A/S, Denmark). Pressure recordings from this were relayed via the Polygraf.

6.4.3 Solutions:

The 2 distal manometry channels were called the 'gastric' channels as these would contain hydrochloric acid during the observation period. The 2 channels proximal to this were called the 'oesophageal' channels as these would be non-acid containing. This dictated the constituents of the 50ml syringes. Three separate study days were undertaken. One acted as a control, one had NA and the last had a NO-generating solution contained in it. A summary of the syringe contents can be seen in Table 6.1.

6.5 Bench-top studies:

We performed bench-top studies to confirm the concentrations and amount of NA and NO delivered by the 3 test solutions through the ports of the apparatus. This was performed at 37°C. NO concentration was measured by a NO probe (World Precision Instruments, Florida, USA) and NA by Greiss reagent. We also assessed the amount of gaseous NO which could be produced *in vitro* by each infusion under physiological conditions.

6.6 Data analysis:

6.6.1 Transient lower oesophageal sphincter relaxations:

TLOSRS were identified from the tracings as prolonged relaxation of the LOS as per Holloway's criteria. TLOSRS are not associated with swallowing either 4 seconds before or 2 seconds after the event. The LOS relaxation is rapid ($\geq 1\text{mmHg/sec}$) and decreases to IGP giving a 'common cavity' event with the oesophagus (nadir pressure $\leq 2\text{mmHg}$). Time to complete relaxation is $\leq 10\text{secs}$. LOS relaxation is longer than that following

swallow-induced relaxation and is often followed by a marked after-contraction (112). Frequency, duration and timing during the infusion of TLOSRS were documented.

6.6.2 Intra-gastric pressure:

Post-prandial IGP, in the upright position, was measured prior to the start of the infusions. Changes in IGP relative to this were analysed at 15 minute intervals throughout the 3 infusions. To do this, a short section (approximately 30 seconds) of the tracing, during proximal stomach inactivity, was taken and analysed as a guide of IGP. This was repeated approximately 15 minutes later on the tracing, or as close to this as possible, as long as there was no obvious contractile activity present.

6.6.3 pH:

The number of pH events with a pH less than 4 were recorded, and also the duration of each event (time taken for pH to rise above 4). This allowed us to calculate a percentage time exposure of pH<4, the average time per reflux episode and also the total number of reflux episodes for each probe under each study condition.

6.6.4 Manometry:

Parameters of the HPZ were made by calculating the mean value from the 4 distal manometry channels. The lower border of the HPZ was identified as the point where the end expiratory pressure remains above that of gastric baseline pressure throughout the full respiratory cycle. The upper border of the HPZ was identified as the point where the pressure drops below

oesophageal baseline pressure in end expiration. The RIP was identified as the point where the pressure deflection decreased in expiration. The MEEP was used as the marker of overall HPZ pressure. This was taken in the trough phase of respiration and calculated relative to the fasting gastric baseline pressure.

Subtracting the mean of the 4 HPZ upper border positions from the mean of the 4 HPZ lower border positions gave a mean total HPZ length for each subject. Subtracting the mean of the 4 RIP values from the previously calculated mean lower border of the HPZ gave a mean length for the abdominal length (AL) of the HPZ for each subject.

6.6.5 Swallowing:

The frequency of swallowing events during the infusions was assessed. Each infusion was divided into the four time quartiles to see if there was any trend between each solution in the pattern of swallowing.

6.6.6 Peristalsis:

To further investigate the acid reflux and clearance data collected, data from the oesophageal manometry ports was collected. The three channels represented the proximal, mid and distal oesophagus. Pressure amplitude of contraction, peristaltic wave velocity and peristaltic wave duration were calculated. Wave velocity was only calculated for the mid and distal oesophagus. All swallows were 'dry' and the data should be interpreted accordingly.

6.7 Statistics:

As each subject was used as his or her own control the following were analysed using a 2-sided paired t-test. Total number of TLOSRS, mean duration of TLOSRS, percentage time pH<4 and total number of reflux events. Pooled data of all reflux events to assess the mean time per reflux episode was analysed using a 2-sided unpaired t-test. ANOVA was also used to examine the changes in IGP during the infusion.

6.8 Blinding:

All studies were carried out in a double-blinded randomised-controlled fashion. Analyses were performed before unblinding of the data took place.

6.9 Ethics:

This study was approved by North Glasgow University NHS Trust Ethics Committee and all subjects gave written informed consent.

6.10 Results:

6.10.1 Manometry:

6.10.1a TLOSRS:

The mean number of TLOSRS per infusion was similar in both the control, mean 3.5 (SD 1.7), and NA groups, mean 3.2 (SD 2.1), but significantly greater in the NO group, mean 5.2 (SD 2.1), ($p<0.01$ & $p<0.0001$ respectively) – see Figure 6.3. The mean duration per TLOSRS was similar in each group, being 20.6 seconds (SD 3.5) for the control, 21.3 seconds (SD 4.7) for NA and 22.9 seconds (SD 4.4) for NO generating – see Figure 6.4.

6.10.1b Temporal relationship of reflux events to TLOSRS:

On no occasion did the pH rise during a TLOSRS. There was, however, no change in pH following 29 of the 177 TLOSRS. 6 occurred during the control runs, in 3 separate patients. Of these 6, 2 happened with a pH less than 4 (mean pH 1.7). 3 occurred during the NA runs, in 2 different patients. 2 occurred with a pH less than 4 (mean pH 2.25). 20 occurred in the NO runs, in 7 different patients. 19 of these events had a pH less than 4 (mean pH 1.4). A total of 52 TLOSRS occurred during the 15 control studies. For 15% of these the distal pH sensor was at a pH less than 4 prior to the TLOSRS. This is not different to the NA arm (21%), ($p < 0.46$), but less than that seen for the NO arm (46%), ($p < 0.0001$ vs control, $p < 0.003$ vs NA).

6.10.1c Quartile assessment of TLOSRS frequency:

Dividing the hour infusion into 15-minute sections allows us to look for trends of TLOSRS between the infusions. The NO group had a significantly higher frequency of TLOSRS than the control group in the first and last quarters ($p < 0.03$ for both). The levels were higher yet again in the middle 2 quarters, but did not reach significance, possibly due to underpowering of the study. Also there was an obvious trend that the frequency of TLOSRS was maintained throughout the infusion. This differed to both the control and NA groups, which showed a trend towards a reduction in TLOSRS frequency as time progressed – see Figure 6.5.

6.10.2 Intra-gastric pressure:

Following complete ingestion of the meal (n=45), IGP was increased by 4.9mmHg (SD 1.3mmHg), ($p<0.00001$). The rise in IGP caused by the meal, measured in the supine position, was assessed for each study day group in turn. The control group had a mean rise of 5.6mmHg (SD 2.3mmHg), which was not different to the NA group (mean 4.9mmHg, SD 2.1mmHg), but was significantly greater than the NO group (mean 4.1mmHg, SD 0.91mmHg), ($p<0.02$). The NA group was not different to the NO group – see Figure 6.6.

Using the immediate post-meal IGP as a reference point, the control group and the NO group showed an increase in IGP over the hour infusion of 1.8mmHg (SD 2.6mmHg), ($p<0.02$) and 3.4mmHg (SD 3.7mmHg), ($p<0.005$) respectively. In contrast the NA group showed a small decrease in IGP (-1.7mmHg, SD 4.6mmHg, $p<0.16$). The NA group was significantly different to the control group at the 30-45 minute period ($p<0.02$ by t-test & $p<0.006$ by ANOVA) and to both the control ($p<0.03$) and NO groups ($p<0.008$) for the 45-60 minute period – (Figure 6.7). Scatter plots of the individual points for the 3rd and 4th quarters relative to the post-meal IGP can be seen in Figures 6.8 & 6.9. A summary of the mean and SD for each IGP arm relative to the post-meal pressure can be seen in Table 6.2. Less compelling data are seen when the IGP relative to the fasting values are used – Table 6.3, Figures 6.10 & 6.11.

6.10.3 Effect of the meal (on single time-point pressure pull-through), (n=45):

Following ingestion of the meal and prior to commencing any test infusions, the total HPZ length (n=45) was 3.48cm (SD 0.63cm) compared to 4.94cm (SD 0.76cm) before the meal ($p<0.0001$). This was mainly due to shortening of the abdominal component of the sphincter from 2.87cm (SD 0.62cm) to 1.80cm (SD 0.59cm) ($p<0.0001$) with less marked shortening of the thoracic component from 2.1cm (SD 0.79cm) to 1.7cm (SD 0.34cm), ($p<0.0001$) (Figure 6.12). The lower border of the HPZ elevated from 47.63cm (SD 2.63cm) to 45.98cm (SD 2.76cm) following the meal ($p<0.0001$), but there was no significant change in the upper border. The RIP elevated from 44.76cm (SD 2.53cm) to 44.17cm (SD 2.49cm), ($p<0.0001$), presumably reflecting slight elevation of the diaphragm by the distended stomach. The meal also produced a fall in the MEEP of the HPZ from 24.5mmHg (SD 9.4mmHg) to 21.2mmHg (SD 8.1mmHg), ($p<0.04$), (Figure 6.13). All of these pressures used fasting IGP as a baseline. A diagram depicting the change in the shape of the HPZ can be seen in Figure 6.14 and a summary in Table 6.4.

6.10.4 Effect of the infusions following meal (on single time-point pressure pull-through), (n=15):

6.10.4a Total LOS length:

Following the control infusion mean LOS length was 4.3cm (SD 0.65cm), which was shorter than the fasting LOS, (mean 5.0cm, SD 0.63cm) ($p<0.002$), but longer than the immediate postprandial LOS values (mean

3.5cm, SD 0.79cm) ($p < 0.002$). There was no difference in the post-infusion LOS comparing control v NA ($p < 0.15$), control v NO generating ($p < 0.11$) and NA v NO generating ($p < 0.83$). See Figure 6.15.

6.10.4b Abdominal sphincter length (AL) (n=15):

Following the control infusion mean AL was 2.2cm (SD 0.56cm). This was shorter than the fasting AL (mean 3.0cm, SD 0.56cm) ($p < 0.00001$) and longer than the postprandial AL (mean 1.8cm, SD 0.70cm) ($p < 0.03$). There was no difference in the post-infusion AL comparing control v NA ($p < 0.45$), control v NO generating ($p < 0.62$) and NA v NO generating ($p < 0.22$). See Figure 6.16.

6.10.4c Maximum end expiratory pressure (n=15):

When the pre and post-meal LOS pressures were analysed within their respective study groups (n=15), the results are less compelling compared to the pooled data (n=45). Post-infusion MEEPs can be compared directly. There is no difference between the MEEPs of the three groups (n=15) fasting. The MEEP was 25.8mmHg (SD 9.1mmHg) after the control solution compared to 20.2mmHg (SD 10.4mmHg) after the NA series ($p < 0.03$). MEEP was 23.7mmHg (SD 11.8mmHg) after NO and not different to the control ($p < 0.31$).

Making comparisons between the post-meal MEEPs and the post-infusion MEEPs reveals no significant differences between any of the three groups. A summary of post-infusion LOS data can be seen in Table 6.5.

6.10.5 pH step-up point relative to HPZ:

A total of 90 fasting pH pull-throughs were performed, using the 2 sequential pH probes, followed by 45 fasting LOS slow-motorised manometry pull-throughs. Plotting the lower and upper borders for each sphincter, the mean position of the pH step-up point in the sphincter can be located and its position relative to the RIP. The mean fasting position of the RIP was $44.76\text{cm} \pm 0.4\text{cm}$ and the mean position of the pH step-up was at $44.97\text{cm} \pm 0.5\text{cm}$. The mean upper border position of the LOS was 42.7cm and the mean lower border of the LOS was 47.6cm . This shows that the fasting pH step-up point was contained well within the high-pressure zone of the LOS and that it was positioned in the abdominal portion of the sphincter - (Figure 6.17). No post-prandial pH pull-through was performed.

6.10.6 Position of the distal pH probe relative to the upper border of the HPZ:

Postprandial HPZ lengths vary from subject to subject. With the distal manometry channel located in the proximal stomach, the distance between the upper border of the HPZ and distal pH probe will also vary. Not only will this vary between each subject but perhaps slightly between study days. The mean distance above the upper border of the HPZ of the distal pH probe was 2.2cm (SD 1.03cm) for the control run, 1.8cm (SD 0.72cm) for the NA run and 2.2cm (SD 0.77cm) for the NO generating arm. Using a 2-sided paired sample t-test there was no significant difference comparing control v NA ($p < 0.11$), control v NO generating ($p < 0.97$) and NA v NO generating ($p < 0.08$) – see Figure 6.18.

6.10.7 Assessment of reflux:

6.10.7a Proximal probe:

There was no significant difference in mean oesophageal acid exposure of the proximal pH probe, being 26.5% (SD 25.5%) for control, 29% (SD 27.4%) for NA and 43.4% (SD 39.7%) for NO generating. The frequency of reflux episodes was also similar. However, the duration for each reflux episode was greater in the NO group, mean 387.6 seconds (SD 544.1 seconds), compared to both NA, mean 107.9 seconds (SD 102.3 seconds), ($p < 0.049$) and control, mean 84.1 seconds (SD 71.2 seconds), ($p < 0.04$). Scatter plots can be seen for the proximal probe data in Figures 6.19, 6.20 & 6.21.

6.10.7b Distal probe:

The control solution produced a mean oesophageal acid exposure of 37.5% (SD 27.9%), NA 36.6% (SD 26.1%) and NO generating 62.2% (SD 33.5%). Control was not different to NA ($p < 0.90$), but NO gave a significantly greater acid exposure compared to both control ($p < 0.03$) and NA ($p < 0.002$). There was no significant difference in the mean number of reflux episodes between the groups, but there was prolongation of reflux in the NO group. The mean duration in the NO group was 574.6 seconds (SD 920.1 seconds), which was almost significantly longer than both the NA, mean 108.4 seconds (SD 104 seconds), ($p < 0.054$) and the control group, mean 122.5 seconds (SD 127.6 seconds), ($p < 0.08$). Scatter plots can be seen for the distal probe data in Figures 6.22, 6.23 & 6.24. A summary of all pH data and statistics can be seen in Tables 6.6 & 6.7 respectively.

6.10.8 Swallowing:

There is no difference between the total number of swallowing events and the three test solutions, either for the whole infusion or individual quartiles. See Tables 6.8a-d.

6.10.9 Peristalsis:

6.10.9a Peristaltic pressure amplitude –

As expected, the peristaltic pressure amplitude, was less in the proximal oesophagus (mean 43mmHg, SD 13.3) compared to both the mid (mean 77mmHg, SD 41.3) and distal oesophagus (mean 91.4mmHg, SD 45.7), for the control solution. This was the same for both test solutions. There were no significant differences between solutions for either overall average pressure amplitude, or each individual quartile. This is true especially for the distal oesophagus, where the reflux was occurring. See tables 6.9a-c, 6.10a-c, 6.11a-c and 6.12.

6.10.9b Peristaltic velocity –

With the first manometry recording channel in the proximal oesophagus, the peristaltic velocity can only be calculated for the mid and distal oesophageal segments. Overall, there was no effect on peristaltic velocity between the three solutions, in either the mid or distal oesophagus. This included analysis of both the entire infusion duration and also each individual quartile. Some of the velocities were calculated as being negative values. This was true for all 3 solutions in the mid oesophagus and the NO group proximally. See Tables 6.13a-c, 6.14a-c and 6.15. This is presumably

due to the way in which the software automatically calculates the velocity and partly due to some incoordination caused by dry swallowing.

6.10.9c Peristaltic duration –

Analysing the infusions as a whole, there was no significant effect on the peristaltic duration between each, for any oesophageal segment. There was a significant prolongation of the duration between Control (5.5 secs) compared to NO (7.2 secs), ($p < 0.02$), in the third quartile. However, as there was no significant difference in the fourth quartile, this is of doubtful importance. See Tables 6.16a-c, 6.17a-c, 6.18a-c and 6.19.

6.10.10 Bench-top *in vitro* NO studies:

A calibrated NO probe indicated that the concentration of NO at the catheter tip at 37°C, following dilution of the solution by the distilled water from the proximal catheter ports was 6µmol/l for the NA solution and 0.86mmol/l for the NO generating solution. This allowed us to calculate the dose of NO and NA given in each study as shown in Table 6.20. We also calculated that the volume of NO gas formed from the NO generating solution was approximately 60mls during the hour infusion and 30mls from the NA solution. See Chapter 5.

6.11 Discussion:

Our studies indicate that upper gastrointestinal physiology may be influenced by the intraluminal administration of NO and NA, both of which are generated when saliva is acidified in the proximal stomach. The situation is complex in that the effects of the two chemicals are different.

The design of our study involved modifying the manometry system to allow us to administer the test solutions within the manometry perfusion solution. As the study involved each subject ingesting a meal we were able to validate the ability of our modified manometry system to detect physiological changes in sphincter morphology induced by the meal. The meal caused shortening of the abdominal portion of the sphincter and a reduction in MEEP. The abdominal portion shortening can be explained by a rise in the intragastric pressure overcoming the pressure of the distal sphincter. However, as the MEEP is referred back to fasting IGP this reflects a true reduction in the MEEP. In addition the RIP was slightly elevated after the meal, probably due to the distended stomach displacing the diaphragm upwards. The rise seen in IGP following the meal (4.9mmHg) is similar to that seen by Kahrilas' group (4mmHg) which was needed to trigger TLOSRS (123).

Our pilot studies and positive control experiments (section 2.4.1) were able to demonstrate a reduction in LOS MEEP. These reductions are recorded at approximately 15 minutes, which is long after the pull-through in this study. We would normally expect a greater LOS relaxation post-meal. This would suggest that LOS relaxation is likely to be hormonally mediated in part, possibly via cholecystokinin (238). Also, the variability in one-off LOS pressure measurements has already been discussed. The reason for not delaying the pull-through in our studies was to maximise the time of the meal in the stomach and thereby increase the chance of observing TLOSRS.

The luminal administration of the NO generating solution and NA without ascorbic acid, influenced the function of the distal oesophagus and GOJ. Compared to the Control, NO caused a significantly increased acid

exposure of the lower oesophagus. In the proximal oesophagus, the duration of each reflux episode was increased significantly by the NO solution. There was no increase in the total number of reflux episodes in either part of the oesophagus. There was no increase in the number of reflux episodes despite there being an increase in the number of TLOSRS. The intra-oesophageal pH was more commonly less than 4 from a previous reflux episode, before a TLOSRS, in the NO arm. This may indicate an overlap of reflux episodes. However, as there is no hard evidence of dysmotility in the lower oesophagus, impaired clearance cannot be blamed directly. A previous study showed that if NO effectiveness was increased, by inhibiting phosphodiesterase V, which degrades cyclic-GMP, lower oesophageal motility and subsequently acid clearance were impaired (179).

We assessed oesophageal motility by looking at the intermittent oesophageal peristalsis during the studies. Although difficult to compare, as it was mainly 'dry' swallows during the tests, there was no obvious difference between the 3 study arms. However, there was a significant increase in the number of TLOSRS following NO. It must be observed that the acid exposure was extremely high, even for the Control arm. This is likely to be due to both the volume of the acidic solution infused and also the presence of the instrumentation within the lumen.

Autonomic upset leading to impaired salivary function was excluded by blood pressure and heart rate monitoring. Perhaps there are other mechanisms involved in clearance. The function of the muscularis mucosa in the oesophagus is little understood and consequently often overlooked. Relaxation of the muscularis mucosae by NO could theoretically cause an impairment of acid clearance, at a level that is not recordable by standard

manometry. Certainly, on the IGP scatter data, the NO data appears to have a bimodal distribution. This identifies either a group more susceptible to the effects of NO, or conversely, a group more resistant. However, on subanalysis of the data that we collected, these 2 populations did not appear to be at different ends of the 'reflux spectrum', for healthy volunteers.

The level of acid exposure at the pH probes is higher than would be expected, even for the control arm of the study. There are several explanations for this. Preventative features of acid reflux rely on the presence of a competent sphincter. Splinting of the LOS with a manometry catheter may have some effect on it's ability to perform. In a similar way, the presence of a 4mm manometry catheter/pH assembly through the length of the oesophagus, possibly impairs the mechanisms that perform acid clearance from the lumen. A single pH catheter in the oesophagus, and indeed even across the LOS, has been shown not to affect LOS function or acid clearance (239). This aside, the catheter is present, and therefore controlled for, in each of our experimental arms.

Taping 2 instruments together raises the potential for capillary action where their lengths make contact. This could give refluxate the opportunity to travel vertically and independent of oesophageal motility. Although it is almost impossible to measure and assess this, with an identical apparatus and meal being used for each experimental arm, this can also be considered controlled for. We have not seen any effect on standard parameters of oesophageal motility ie: peristaltic amplitude, wave velocity and duration, by any of the solutions, indicating that the increased acid exposure seen in the NO arm of the study may be caused by more subtle mechanisms.

Finally, the volume of gastric acid secreted is known to increase after a meal, in response to the release of gastrin (240). Submaximal stimulation of gastric acid secretion with intravenous pentagastrin (0.05mcg/kg/hr), in *H. pylori* negative subjects will increase the basal volume from 40-80mls/hour, to between 160-300 mls/hour. This will be secreted relatively uniformly by the acid secreting mucosa, which extends down through the body of the stomach. During our experiment, we can safely assume that the stomach secretes it's own acid, in addition to the 400mls that is infused into the proximal stomach. This extra volume may explain the slight increase in IGP seen in the Control and NO arms during the study.

Our research group has previously shown the presence of an unbuffered acid pocket in the proximal stomach following a meal (23). It is likely that a modest proportion of the infused acid runs off to the distal stomach. However, we are also likely to be exaggerating the acid pocket for each study arm. The amount of ascorbic acid is certainly higher than we would normally expect to find at the cardia (fasting mean in healthy volunteers 13.0 μ mol/l – SEM 6.1 μ mol/l) (241). What would normally be a limited exhaustive supply, with higher fasting levels in the gastric body and distal stomach than at the cardia, is now present in excess.

There was a much greater number of reflux events than TLOSRS. Cumulative data shows 177 reflux events for the 15 subjects during the control studies compared to 52 TLOSRS. There were 190 cumulative reflux events for the 15 subjects in the NA studies compared to 47 TLOSRS. There were 184 cumulative reflux events documented for the 15 subjects in the NO studies compared to 78 TLOSRS. 19 of these 78 TLOSRS could not have a reflux event documented against them as the pH was already less than 4 and

did not reduce any further. This also means that many reflux events occurred outwith a TLOS. It is possible that they occurred due to loss of sphincter tone or episodes that did not meet the criteria of a TLOS. Without a manometry sleeve device across the LOS it would be difficult to say with total certainty whether or not this was the case.

Despite the increased frequency of TLOSs and oesophageal acid exposure we did not observe any changes in the LOS pressure. However, the latter was measured only at a single time point and several minutes after discontinuing the NO infusion. It is of note that the abnormalities detected, i.e. TLOSs and acid exposure, were detected by continuous monitoring during the infusion. It is therefore possible that changes in sphincter pressure have been missed due to the technical impossibilities of monitoring this during NO administration. NO relaxes the LOS during peristalsis and this is a transient phenomenon (137).

It is important to remember that TLOSs require relaxation of both the LOS and the crural diaphragm. It is highly unlikely that luminal NO would be able to permeate and have a direct effect on the diaphragmatic musculature. We have already considered the possibility that the NO containing solution might trigger TLOSs due to the gas coming out of solution and causing gastric distension. However, our extensive benchtop studies, simulating our intragastric infusions, demonstrated that only approximately 60mls of gas are produced by the NO generating solution and 30mls by the NA solution throughout the one hour infusion period. Considering that each swallow introduces 15mls of air into the stomach (13), it seems unlikely that the small amount of gas produced through the one hour infusion will have exerted any significant effect. It is possible that NO is triggering TLOSs by altering the

motility and morphology of the cardia region and possibly affecting the muscularis mucosae, which will be the smooth muscle closest to the lumenally generated NO. The muscularis mucosae has been shown to relax in response to micromolar concentrations of NO *in vitro* (102).

It is also possible that locally high concentrations of NO may be able to cross through the mucosa of the proximal stomach and exert some effect on afferent receptors involved in TLOS co-ordination. We have shown that NO can cross through the thickness of a rat's stomach *in vitro* (unpublished laboratory work). Two weaknesses of this *in vitro* study are the absence of tissue blood flow and also surface mucous secretion. There are many perforating blood vessels present in the submucosa of the GOJ (38) and certainly the potential for this to 'mop-up' any absorbed NO, particularly by haemoglobin, could be one factor in reducing any local effect on the major muscle bulk of the LOS. This may explain the lack of effect seen on the MEEP within the NO arm. Nitric oxide at these high concentrations must somehow be making conditions more favourable in the proximal stomach either with regards to stretch or tone in the stomach wall or perhaps by interacting with a population of NO receptors that trigger the TLOS cascade.

The NA solution did not cause increased oesophageal acid exposure but caused a significant reduction in IGP during the post-prandial period, compared to the control arm. As previously stated, NA solution reacts with thiols to form nitrosothiols (197). The latter are relatively stable and long-lived, and act as NO donors (242). The reduced IGP following the NA may be due to these nitrosothiols exerting an effect on the tone of the gastric wall.

NO will have a much shorter half-life and its effect may be limited to the muscularis mucosae which is much closer to the luminal surface.

In some of the data (Figure 6.8), the scatter plots suggest a 'dual effect' for the NO group, with a mild bi-modal population. We also know that NA exists as approximately 1% NO under these conditions. If there is a NO effect produced in the NA arm, by the proportion expected from a NA solution, it is either too small to see or abolished by the other component of NA's action.

6.12 Clinical significance:

The pressure differential in IGP between Control and NA during the infusions is 3.5mmHg. This is a very important finding. Intra-gastric pressure directly acts on the abdominal portion of the sphincter. It is this lower pressure that may potentially allow the cardia to 'reassemble'. This degree of gastric relaxation would allow a SMPT to 'detect' the abdominal sphincter sooner than if the IGP were higher. Whether the underlying mechanism be one of gastric relaxation, there is no doubt that this in fact may be a protective mechanism against reflux disease. If retaining food within the stomach, by gastric relaxation, NA may in fact be protective against reflux disease by prolonging food buffering of secreted gastric acid.

It is further borne out looking at the frequency of TLOSRS. Gastric relaxation, with subsequent changes to the abdominal sphincter may generate less afferent stimulation to trigger TLOSRS. Sphincter parameters post-infusion (Table 6.5 & Figure 6.15) showed that the NA studies have more significant gains in abdominal sphincter length compared to both Control and NO. Could it be a drop in IGP that allows this to occur?

It is important to relate the *concentration* and *amount* of chemicals administered in our study with those occurring physiologically with swallowing saliva. In the fasting state the *concentration* of nitrite in saliva varies from approximately 50-600 $\mu\text{moles/litre}$ and rises several fold following ingestion of nitrate in the diet. The *concentration* of nitrite administered in this study is therefore at the upper limit of the range occurring in swallowed saliva after a nitrate-rich meal. The *amount* of nitrate ingested each day in food is up to 2 millimoles (243) with approximately 25% of this (500 μmoles) being secreted in saliva in the post-prandial period (167). The *amount* of nitrate reduced to nitrite by buccal bacteria is highly variable, ranging between 5% and 90%. Based on these calculations, the *amount* of nitrite administered during our one-hour infusion approximates to the upper limit of the amount delivered during a days' post-prandial period.

Our studies indicated that the presence of ascorbic acid, which converts the NA to NO, influenced the effects of the solution on both gastric and oesophageal function, within healthy subjects. It is therefore important to consider factors that determine intragastric ascorbic acid availability. Ascorbic acid is actively secreted in gastric juice (244). It's median intragastric value in subjects with a healthy stomach is 250 $\mu\text{mol/l}$ and is substantially lower in subjects with *H.pylori* infection (245), atrophic gastritis (216) and those of black ethnicity (246). Eating will considerably enhance the concentration of ascorbic acid as it is present in substantial concentrations in many foodstuffs. Ingesting a diet rich in both nitrates and also anti-oxidants may enhance any effects of physiological reflux and indeed predispose to inflammation, Barrett's and cancer.

There is some evidence that *H.pylori* may play a protective role against reflux disease (247;248). Carditis and intestinal metaplasia at the GOJ is more common in *H.pylori* positives than negatives (249), but this trend may be changing. This could have the effect of reducing the amount of acid-secreting mucosa available in a post-prandial 'acid-pocket'. As this is assumed to be the area from which oesophageal acid reflux is generated, it is clearly important. Also, there is an association of reduced levels of ascorbic acid in plasma of patients of lower socio-economic class (250).

These can be linked in two ways. Firstly, the lower incidences of reflux disease and GOJ cancer in lower social classes mirrors their reduced ability to secrete ascorbic acid. They are also more likely to be *H.pylori* positive (251) with a greater predisposition to developing gastric cancer, probably related to an atrophic gastritis (252).

Another piece of evidence that picks out ascorbic acid as playing a key role in acid reflux is the association between black skin colour and low levels of gastric ascorbic acid (246). This correlates to a low prevalence of both GORD and oesophageal adenocarcinoma. Clearly, in these situations where there is less ascorbic acid there is likely to be more NA at the cardia. This in itself may be a protective mechanism against GORD. One previous study investigated the effect of oral nitrate supplements on oesophageal function in *H.pylori* negative healthy volunteers and GORD patients. Fasting basal LOS pressure measured using a sleeve did not appear to be affected in either group by the addition of nitrate to the diet (159).

TLOS provocation was performed by rapid injection of 750mls of air into the gastric body. Therefore, the majority of the intragastric volume could be expelled with belching, which differs to our meal. It is difficult to comment

on whether salivary nitrite levels were recorded at the appropriate time after a dose or not. It is also likely that the GORD patients in this study had intact oesophageal mucosa due to their prior therapy, and there is also no comment on the proportion with columnar-lined oesophagus. These are two important sub-sets of patients that require further evaluation.

Our current studies serve as a proof of the concept, that the chemicals generated when nitrite-rich saliva encounters gastric acid can influence gastro-oesophageal function. We have generated post-prandial luminal NO, in the correct location, probably at it's physiological upper limit. The question now remains as to where the threshold lies for such effects to take place. Does it vary between healthy volunteers and those with upper GI disease?

Amongst this group, do patients with a hiatus hernia differ to those without a hernia but with oesophagitis? Also, what is the effect on acid clearance in those with Barrett's oesophagus? Does luminal NO have a different effect, or different threshold for its effect, in the presence of a columnar-lined oesophagus? Also, does NA have a protective role in healthy volunteers, which is lost in GORD sufferers? It is now important to investigate whether this luminal chemistry contributes to disordered motility of the stomach and GOJ. The information from this study may allow us to target various areas of this pathway, including the amount of ingested nitrate and eliminating buccal bacteria.

6.13 Grant support:

This work was funded in part by a North Glasgow University Hospitals NHS Trust research endowment fund.

Table 6.1: Concentration of chemicals in syringes on the different study day.

Gastric channels	Oesophageal channels
Control study day	
Thiocyanate – 1mM	Thiocyanate – 1mM
Hydrochloric acid – pH 1.0	
EDTA – 1mM	
Ascorbic Acid – 5Mm	
Nitrous Acid study day	
Thiocyanate – 1mM	Thiocyanate – 1mM
Hydrochloric acid – pH 1.0	Nitrite – 1.6mM
EDTA – 1mM	
Nitrite – 1.6mM	
NO-generating study day	
Thiocyanate – 1mM	Thiocyanate – 1mM
Hydrochloric acid – pH 1.0	Nitrite – 1.6mM
EDTA – 1mM	
Nitrite – 1.6mM	
Ascorbic Acid – 5mM	

Table 6.2: Mean pressure changes in IGP during the infusions, relative to the immediate post-meal IGP. Pressure in mmHg with standard deviation. NA – nitrous acid, NO – nitric oxide.

	2 nd Quarter	3 rd Quarter	4 th Quarter
Control	1.5 (2.1)	1.5 (2.9)	1.8 (2.6)
Acidified Nitrite	0.26 (2.5)	-1.7 (4.6)	-1.2 (5.0)
NO	1.4 (4.4)	0.9 (4.1)	3.4 (3.7)

Table 6.3: Changes in IGP caused by the meal and during the infusions. All values are relative to fasting IGP. Pressure in mmHg with standard deviation.

	Post-meal	2 nd Quarter	3 rd Quarter	4 th Quarter
Control	5.6 (2.3)	7.1 (3.8)	7.1 (4.1)	7.4 (4.3)
Acidified Nitrite	4.9 (2.1)	5.2 (2.7)	3.6 (2.6)	4.2 (2.2)
NO	4.1 (0.9)	5.6 (4.5)	5.4 (3.6)	7.6 (4.0)

Table 6.4: Effect of meal ingestion on the parameters of the LOS compared to the fasting state, (n=45).

LOS parameter	Fasted Mean (SD)	Post-meal Mean (SD)	P value <
Total LOS length / cm	4.94 (0.76)	3.48 (0.63)	0.0001
Upper border / cm	42.69 (2.36)	42.50 (2.43)	0.18
Respiratory inversion point / cm	44.76 (2.53)	44.17 (2.49)	0.0001
Lower border / cm	47.63 (2.61)	45.98 (2.76)	0.0001
Thoracic length LOS / cm	2.1 (0.79)	1.7 (0.34)	0.0001
Abdominal length LOS / cm	2.87 (0.62)	1.80 (0.59)	0.0001
Maximum end expiratory pressure / mmHg	24.5 (9.4)	21.2 (8.1)	0.04
Intragastric pressure / mmHg	0	4.9 (1.3)	0.00001

Table 6.5: Summary of high pressure zone (HPZ) morphology.

	Total HPZ length / cm			Abdominal sphincter length / cm			Maximum end expiratory pressure / mmHg		
	Control	NA	NO	Control	NA	NO	Control	NA	NO
Fasted	5.0 (0.63)	4.9 (0.76)	4.9 (0.91)	3.0 (0.56)	2.9 (0.61)	2.8 (0.7)	23.0 (8.3)	26.3 (11.0)	24.3 (9.1)
Post-meal	3.5 (0.79)	3.5 (0.48)	3.4 (0.65)	1.8 (0.70)	1.8 (0.42)	1.8 (0.64)	21.7 (9.1)	21.4 (8.2)	20.5 (7.5)
Post-infusion	4.3 (0.65)	4.0 (0.82)	4.0 (0.68)	2.2 (0.56)	2.3 (0.63)	2.1 (0.43)	25.8 (9.1)	20.2 (10.4)	23.7 (11.8)

(n=15 for all categories). Data displayed as mean values with standard deviation in brackets.

NA = Nitrous Acid, NO = Nitric Oxide generating

Table 6.6: Summary of pH data (n=15 for all categories).

pH probe	Percentage time pH<4			Total number of reflux episodes			Duration per reflux episode / seconds		
	Control	NA	NO	Control	NA	NO	Control	NA	NO
Proximal	26.5 (25.5)	29.0 (27.4)	43.4 (39.7)	7.3 (7.1)	8.1 (7.2)	8.0 (7.6)	84.1 (71.2)	107.9 (102.3)	387.6 (544.1)
Distal	37.5 (27.9)	36.6 (26.1)	62.2 (33.5)	11.8 (5.5)	12.7 (5.1)	12.3 (10.8)	122.5 (127.6)	108.4 (104)	574.6 (920.1)

Data displayed as mean values. Standard deviation in brackets.

NA = Nitrous Acid, NO = Nitric Oxide generating

Table 6.7: Paired t-test statistics for reflux data displayed in Table 6.6.

		Percentage time pH<4 / p value <	Total number of reflux episodes / p value <	Duration per reflux episode / p value <
Proximal	C v NA	0.66	0.78	0.31
	C v NO	0.07	0.81	0.04
	NO v NA	0.08	0.98	0.049
Distal	C v NA	0.90	0.66	0.73
	C v NO	0.03	0.88	0.08
	NO v NA	0.002	0.90	0.054

C = Control, NA = Nitrous acid, NO = Nitric Oxide

Table 6.8a: Comparison of the number of swallowing events for each solution over the length of the infusion, and each quartile in turn (n=15).

Quarter of infusion / Mean number of swallows							
	1st	2nd	3rd	4th	Mean / Total	Range	SD
Control	13.5	12.4	11.4	11.4	48.7	32-92	16.0
Nitrous Acid	13.3	12.7	11.4	10.4	47.8	29-64	12.9
Nitric Oxide	12.8	12.1	13.2	11.1	49.1	31-87	17.7

Table 6.8b: Comparison of total number of swallows (paired t-tests, n=15).

t-test	p value
Control v Nitrous Acid	0.69
Control v Nitric Oxide	0.70
Nitrous Acid v Nitric Oxide	0.41

Table 6.8c: Inter-quartile comparison of swallowing frequency for each solution (paired t-tests, n=15).

	Q1vQ2	Q1vQ3	Q1vQ4	Q2vQ3	Q2vQ4	Q3vQ4
Control	0.18	0.046	0.04	0.37	0.36	1.0
Nitrous Acid	0.53	0.08	0.02	0.04	0.02	0.16
Nitric Oxide	0.33	0.76	0.15	0.30	0.32	0.04

Table 6.8d: Comparison of the number of swallows for each quartile between solutions (paired t-tests, n=15).

t-test	P value			
	Q1	Q2	Q3	Q4
Control v Nitrous Acid	0.82	0.77	0.85	0.39
Control v Nitric Oxide	0.72	0.40	0.93	0.57
Nitrous Acid v Nitric Oxide	0.73	0.68	0.18	0.63

Table 6.9a: Comparison of the peristaltic pressure amplitude for the proximal oesophageal segment, for each solution and quartile in turn (n=15).

Proximal oesophagus:

	Quarter of infusion / mean pressure amplitude (mmHg)				Mean / Total	Range	SD
	1 st	2 nd	3 rd	4 th			
Control	44.2	42.9	42.8	46.4	43.0	32-57	13.3
Nitrous Acid	45.5	54.9	38.5	42.8	45.4	32-66	11.2
Nitric Oxide	47.6	44.7	47.1	47.1	46.7	26-57	9.4

Table 6.9b: Comparison of the peristaltic pressure amplitude between quartiles for each solution (paired t-tests, n=15).

	Q1vQ2	Q1vQ3	Q1vQ4	Q2vQ3	Q2vQ4	Q3vQ4
Control	0.83	0.99	0.95	0.85	0.79	0.96
Nitrous Acid	0.20	0.22	0.63	0.003	0.06	0.27
Nitric Oxide	0.45	0.90	0.93	0.66	0.62	0.99

Table 6.9c: Comparison of the peristaltic pressure amplitude for each corresponding quartile, between solutions (paired t-tests, n=15).

	p value			
t-test	Q1	Q2	Q3	Q4
Control v Nitrous Acid	0.70	0.11	0.45	0.76
Control v Nitric Oxide	0.65	0.89	0.51	0.45
Nitrous Acid v Nitric Oxide	0.09	0.06	0.24	0.56

Table 6.10a: Comparison of the peristaltic pressure amplitude in the mid-oesophageal segment, for each solution and quartile in turn (n=15).

Mid-oesophagus:

	Quarter of infusion / mean pressure amplitude (mmHg)				Mean / Total	Range	SD
	1 st	2nd	3rd	4 th			
Control	80.3	74.9	85.5	81.2	77.0	20-175	41.3
Nitrous Acid	78.9	83.3	71.6	71.9	76.4	45-140	27.7
Nitric Oxide	66.0	75.7	77.8	72.0	72.9	28-137	30.8

Table 6.10b: Comparison of the peristaltic pressure amplitude between quartiles for each solution (paired t-tests, n=15).

	Q1vQ2	Q1vQ3	Q1vQ4	Q2vQ3	Q2vQ4	Q3vQ4
Control	0.19	0.95	0.50	0.11	0.40	0.61
Nitrous Acid	0.62	0.36	0.31	0.29	0.29	0.96
Nitric Oxide	0.26	0.23	0.44	0.59	0.61	0.38

Table 6.10c: Comparison of the peristaltic pressure amplitude for each corresponding quartile, between solutions (paired t-tests, n=15).

t-test	p value			
	Q1	Q2	Q3	Q4
Control v Nitrous Acid	0.92	0.14	0.56	0.53
Control v Nitric Oxide	0.04	0.72	0.77	0.57
Nitrous Acid v Nitric Oxide	0.07	0.63	0.83	0.93

Table 6.11a: Comparison of the peristaltic pressure amplitude for the distal oesophageal segment, for each solution and quartile in turn (n=15).

Distal oesophagus:

	Quarter of infusion / mean pressure amplitude (mmHg)				Mean / Total	Range	SD
	1 st	2nd	3rd	4 th			
Control	52.4	46.4	57.4	47.7	91.4	21-170	45.7
Nitrous Acid	84.0	103.4	91.9	83.4	90.7	61-134	29.6
Nitric Oxide	77.6	87.7	98.3	84.6	87.1	45-155	38.5

Table 6.11b: Comparison of the peristaltic pressure amplitude between quartiles for each solution (paired t-tests, n=15).

	Q1vQ2	Q1vQ3	Q1vQ4	Q2vQ3	Q2vQ4	Q3vQ4
Control	0.14	0.21	0.60	0.49	0.20	0.27
Nitrous Acid	0.02	0.21	0.92	0.18	0.07	0.15
Nitric Oxide	0.17	0.05	0.46	0.07	0.62	0.06

Table 6.11c: Comparison of the peristaltic pressure amplitude for each corresponding quartile, between solutions (paired t-tests, n=15).

t-test	P value			
	Q1	Q2	Q3	Q4
Control v Nitrous Acid	0.88	0.40	0.94	0.73
Control v Nitric Oxide	0.25	0.17	0.96	0.53
Nitrous Acid v Nitric Oxide	0.60	0.12	0.86	1.0

Table 6.12: Overall comparison of peristaltic pressure amplitudes between solutions (paired t-tests, n=15).

	p value		
	Proximal oesophagus	Mid- oesophagus	Distal oesophagus
Control v Nitrous Acid	0.56	0.89	0.90
Control v Nitric Oxide	0.52	0.58	0.59
Nitrous Acid v Nitric Oxide	0.47	0.42	0.40

Table 6.13a: Comparison of the peristaltic velocity for the mid-oesophageal segment, for each solution and quartile in turn (n=15).

Mid-oesophagus:

	Quarter of infusion / mean peristaltic velocity (cm/sec)				Mean / Total	Range	SD
	1st	2nd	3rd	4 th			
Control	1.8	5.8	6.0	3.3	4.0	-0.4-11.7	3.5
Nitrous Acid	4.3	0.6	9.5	1.1	3.9	-3.2-10.4	4.6
Nitric Oxide	0.9	1.9	5.0	5.0	3.2	-0.8-9.6	2.6

Table 6.13b: Comparison of the peristaltic velocity for each quartile, of each solution, in turn (paired t-tests, n=15).

	Q1vQ2	Q1vQ3	Q1vQ4	Q2vQ3	Q2vQ4	Q3vQ4
Control	0.07	0.40	0.49	0.94	0.09	0.47
Nitrous Acid	0.03	0.38	0.20	0.15	0.87	0.27
Nitric Oxide	0.93	0.09	0.23	0.13	0.16	0.99

Table 6.13c: Comparison of the peristaltic velocity comparison for each corresponding quartile, between solutions (paired t-tests, n=15).

	p value			
t-test	Q1	Q2	Q3	Q4
Control v Nitrous Acid	0.24	0.003	0.50	0.49
Control v Nitric Oxide	0.65	0.02	0.54	0.21
Nitrous Acid v Nitric Oxide	0.19	0.32	0.87	0.38

Table 6.14a: Comparison of the peristaltic velocity for the distal oesophageal segment, for each solution and quartile in turn (n=15).

Distal oesophagus:

	Quarter of infusion / mean peristaltic velocity (cm/sec)				Mean / Total	Range	SD
	1st	2nd	3rd	4 th			
Control	4.4	7.1	4.3	4.1	4.4	2.6-7.9	2.1
Nitrous Acid	6.6	9.5	6.0	4.4	6.6	2.1-29.1	6.6
Nitric Oxide	5.2	4.1	4.6	9.5	5.8	-7.4-25.6	9.8

Table 6.14b: Comparison of the peristaltic velocity for each quartile, of each solution, in turn (n=15).

	Q1vQ2	Q1vQ3	Q1vQ4	Q2vQ3	Q2vQ4	Q3vQ4
Control	0.37	0.75	0.05	0.38	0.06	0.19
Nitrous Acid	0.59	0.79	0.07	0.36	0.34	0.34
Nitric Oxide	0.90	0.79	0.44	0.96	0.57	0.43

Table 6.14c: Comparison of the peristaltic velocity comparison for each corresponding quartile, between solutions (paired t-tests, n=15).

t-test	p value			
	Q1	Q2	Q3	Q4
Control v Nitrous Acid	0.33	0.19	0.46	0.10
Control v Nitric Oxide	0.72	0.88	0.42	0.26
Nitrous Acid v Nitric Oxide	0.38	0.69	0.44	0.44

Table 6.15: Overall comparison of peristaltic wave velocity, between solutions (paired t-tests, n=15).

	p value	
	Mid-oesophagus	Distal oesophagus
Control v Nitrous Acid	0.93	0.26
Control v Nitric Oxide	0.32	0.82
Nitrous Acid v Nitric Oxide	0.82	0.70

Table 6.16a: Comparison of the peristaltic duration for the proximal oesophageal segment, for each solution and quartile in turn (n=15).

Proximal oesophagus:

	Quarter of infusion / mean duration of peristalsis (secs)				Mean / Total	Range	SD
	1 st	2nd	3rd	4th			
Control	3.9	4.2	5.2	5.3	4.2	0.4-6.7	1.7
Nitrous Acid	4.3	5.2	4.4	4.3	4.6	2.9-5.9	1.3
Nitric Oxide	5.5	4.2	5.2	5.1	5.0	2.8-7.8	1.8

Table 6.16b: Comparison of the peristaltic duration between quartiles, for each solution in turn (paired t-tests, n=15).

	Q1vQ2	Q1vQ3	Q1vQ4	Q2vQ3	Q2vQ4	Q3vQ4
Control	0.44	0.04	0.79	0.25	0.60	0.08
Nitrous Acid	0.39	0.87	0.97	0.39	0.31	0.83
Nitric Oxide	0.20	0.76	0.60	0.02	0.17	0.88

Table 6.16c: Comparison of the peristaltic duration between solutions for each quartile in turn (paired t-tests, n=15).

t-test	p value			
	Q1	Q2	Q3	Q4
Control v Nitrous Acid	0.37	0.31	0.46	0.44
Control v Nitric Oxide	0.11	0.84	0.90	0.02
Nitrous Acid v Nitric Oxide	0.31	0.32	0.42	0.43

Table 6.17a: Comparison of the peristaltic duration for the mid-oesophageal segment, for each solution and quartile in turn (n=15).

Mid-oesophagus:

Quarter of infusion / mean duration of peristalsis (secs)							
	1 st	2nd	3rd	4th	Mean / Total	Range	SD
Control	4.8	5.0	5.6	5.4	4.8	2.3-8.3	1.6
Nitrous Acid	5.0	5.0	4.9	4.7	4.9	3.6-6.4	0.8
Nitric Oxide	5.4	4.8	5.2	5.0	5.1	3.1-6.8	1.4

Table 6.17b: Comparison of the peristaltic duration between quartiles, for each solution in turn (paired t-tests, n=15).

	Q1vQ2	Q1vQ3	Q1vQ4	Q2vQ3	Q2vQ4	Q3vQ4
Control	0.92	0.09	0.24	0.13	0.32	0.02
Nitrous Acid	0.95	0.88	0.51	0.85	0.41	0.72
Nitric Oxide	0.27	0.67	0.41	0.14	0.51	0.71

Table 6.17c: Comparison of the peristaltic duration between solutions for each quartile in turn (paired t-tests, n=15).

	p value			
t-test	Q1	Q2	Q3	Q4
Control v Nitrous Acid	0.72	0.40	0.58	0.42
Control v Nitric Oxide	0.49	0.93	0.90	0.17
Nitrous Acid v Nitric Oxide	0.47	0.39	0.94	0.80

Table 6.18a: Comparison of the peristaltic duration for the distal oesophageal segment, for each solution and quartile in turn (n=15).

Distal oesophagus:

	Quarter of infusion / mean duration of peristalsis (secs)				Mean / Total	Range	SD
	1st	2nd	3rd	4th			
Control	5.6	5.4	5.5	7.4	5.7	2.9-7.5	1.5
Nitrous Acid	4.9	6.5	5.8	5.4	5.6	2.7-8.6	1.4
Nitric Oxide	6.5	6.0	7.2	6.7	6.6	3.4-8.9	1.7

Table 6.18b: Comparison of the peristaltic duration between quartiles, for each solution in turn (paired t-tests, n=15).

	Q1vQ2	Q1vQ3	Q1vQ4	Q2vQ3	Q2vQ4	Q3vQ4
Control	0.92	0.79	0.33	0.74	0.32	0.38
Nitrous Acid	0.06	0.14	0.27	0.57	0.25	0.35
Nitric Oxide	0.52	0.35	0.77	0.06	0.23	0.29

Table 6.18c: Comparison of the peristaltic duration between solutions for each quartile in turn (paired t-tests, n=15).

t-test	p value			
	Q1	Q2	Q3	Q4
Control v Nitrous Acid	0.14	0.48	0.94	0.95
Control v Nitric Oxide	0.51	0.55	0.02	0.12
Nitrous Acid v Nitric Oxide	0.08	0.42	0.18	0.11

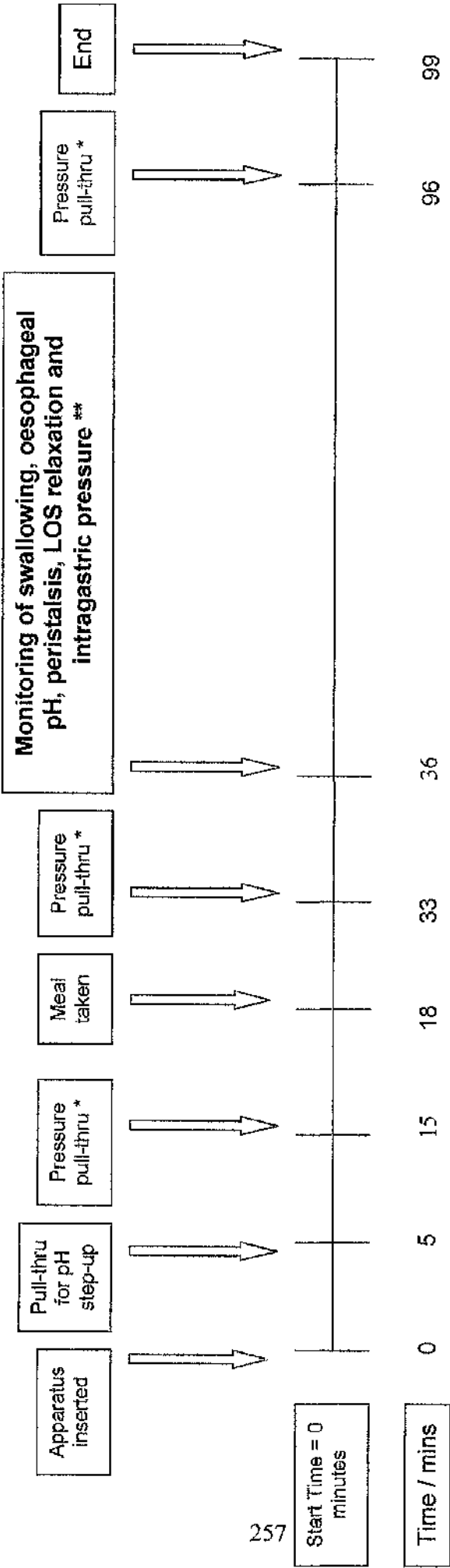
Table 6.19: Overall comparison of peristaltic duration between the solutions (paired t-tests, n=15).

	p value		
	Proximal oesophagus	Mid-oesophagus	Distal oesophagus
Control v Nitrous Acid	0.37	0.78	0.96
Control v Nitric Oxide	0.15	0.57	0.07
Nitrous Acid v Nitric Oxide	0.58	0.85	0.16

Table 6.20: Calculation of NO delivery over one hour from the test solutions. Catheter concentration of NO was performed by the Greiss reaction and takes into account dilution from proximal channel distilled water.

Test solution	Volume delivered litres	NO concentration millimoles/litre	NO load millimoles/hour	Nitrous Acid concentration millimoles/litre	Nitrous Acid load millimoles/hour
NO generating	0.4	0.86	0.344	0	0
Nitrous Acid	0.4	0.006	0.0024	1.6	0.64

Figure 6.1: Illustration of the details of the protocol. Protocol order, infusion solutions, patient posture and relative pressure references are outlined.



Legend:

* Pressure relative to fasting intragastric pressure

** Pressure relative to upright -posture, post-meal intragastric pressure at start of the infusion

Patient posture:

Patient sits upright for pH pull-through, eating the meal and during post-meal monitoring

Patient lays flat for pressure pull-through analysis

Infusion solutions:

No infusion during pH pull-through or eating of the meal.

Distilled water through all 8 lumen for pressure pull-throughs

Distilled water through proximal 4 lumen and test solutions through distal 4 channels during 60 minute post-meal monitoring

Figure 6.2: Location of the pH sensors in relation to the manometry catheter ports.

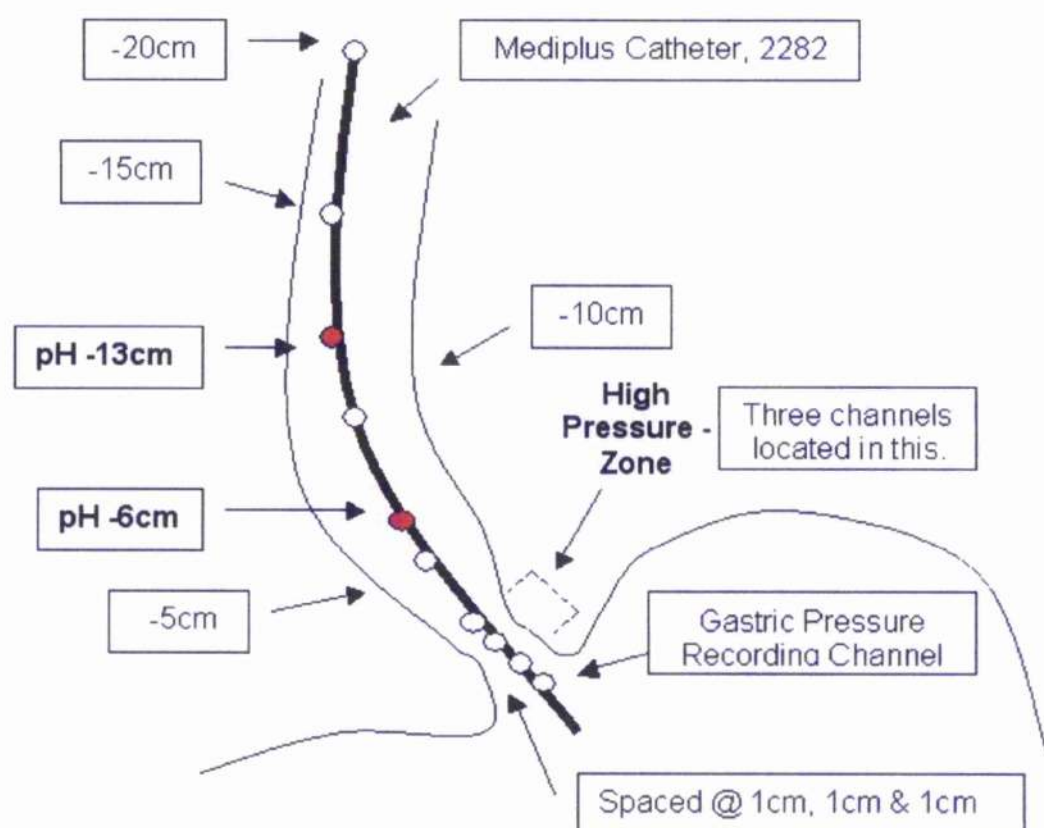


Figure 6.3: Frequency of TLOSRS during the infusion period. Bars indicate median values. Lines join each individual Subject's results.

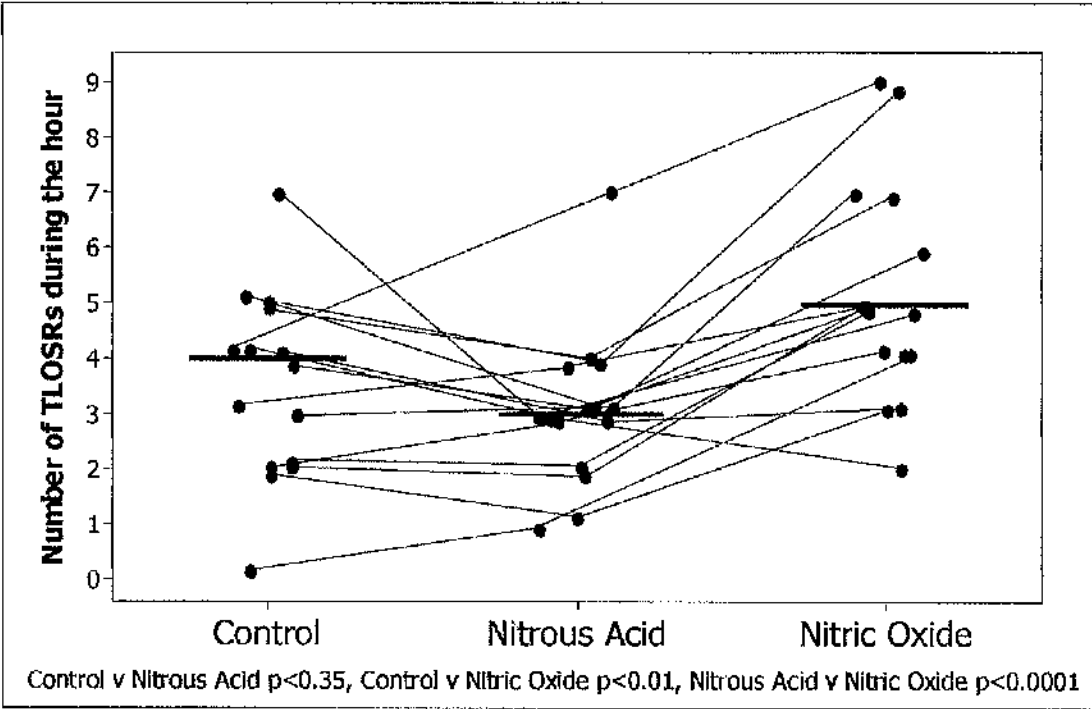


Figure 6.4: Mean duration of TLOSRS for each subject with each infusion. Bars indicate mean values.

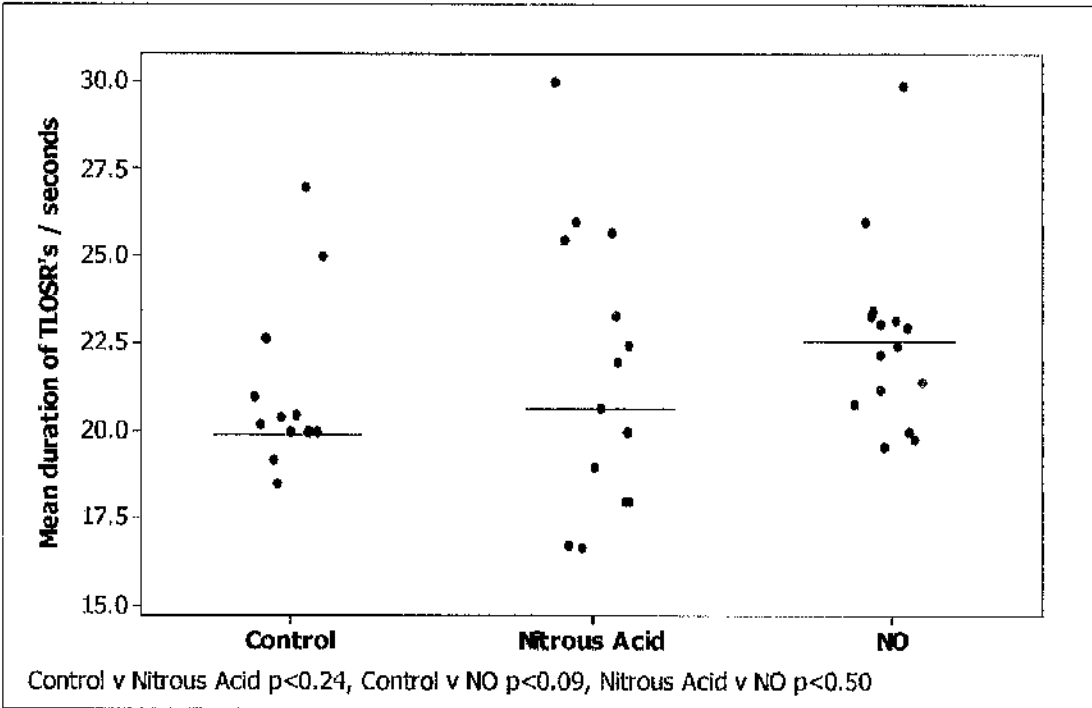


Figure 6.5: Demonstration of TLOSr frequency for each quarter of the three experimental infusions. Error bars indicate standard deviation of the sample. p values represent Control v NO, paired t-tests, n=15.

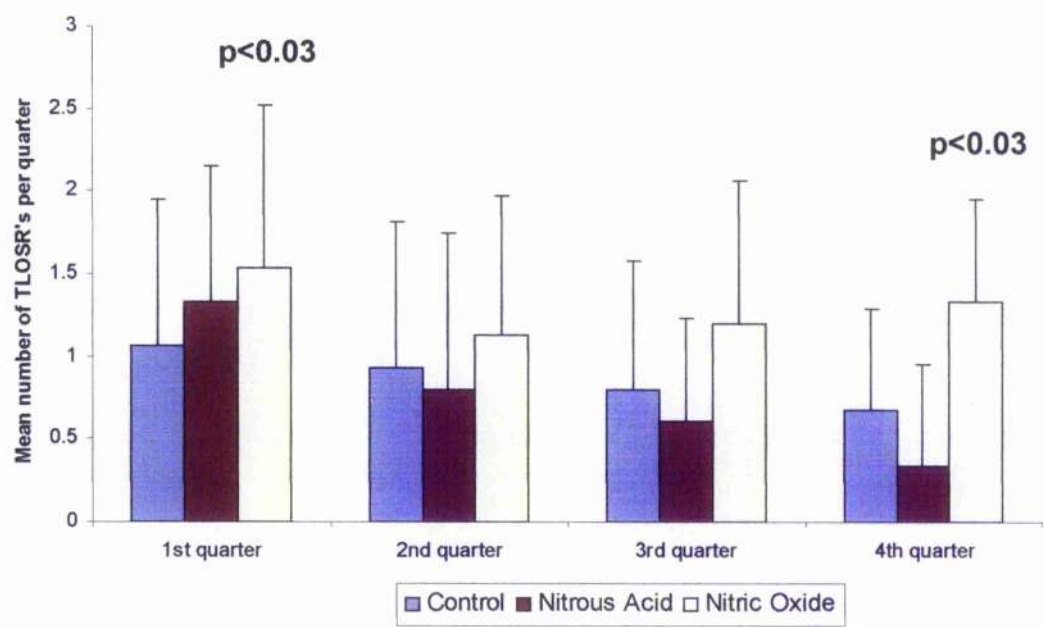


Figure 6.6: Rise in intragastric pressure (mmHg) caused by meal ingestion, for each of the three study days. Bars indicate mean values.

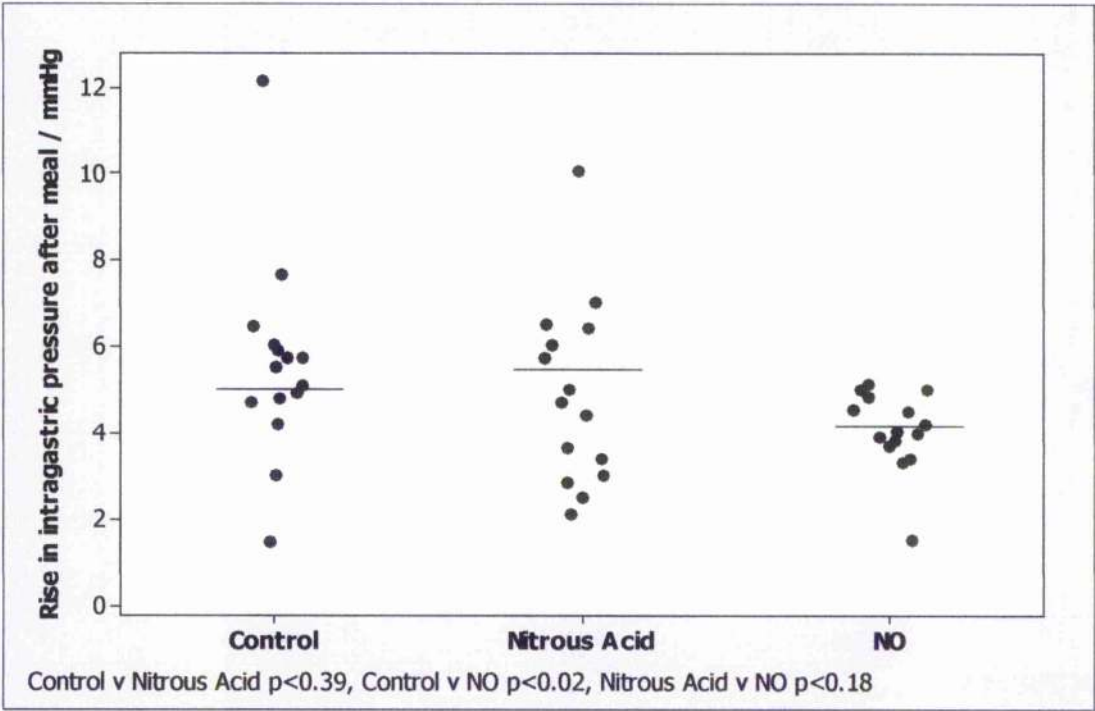


Figure 6.7: Changes in intra-gastric post-meal pressure throughout the infusions. Time on X-axis. Points are mean values (n=15).

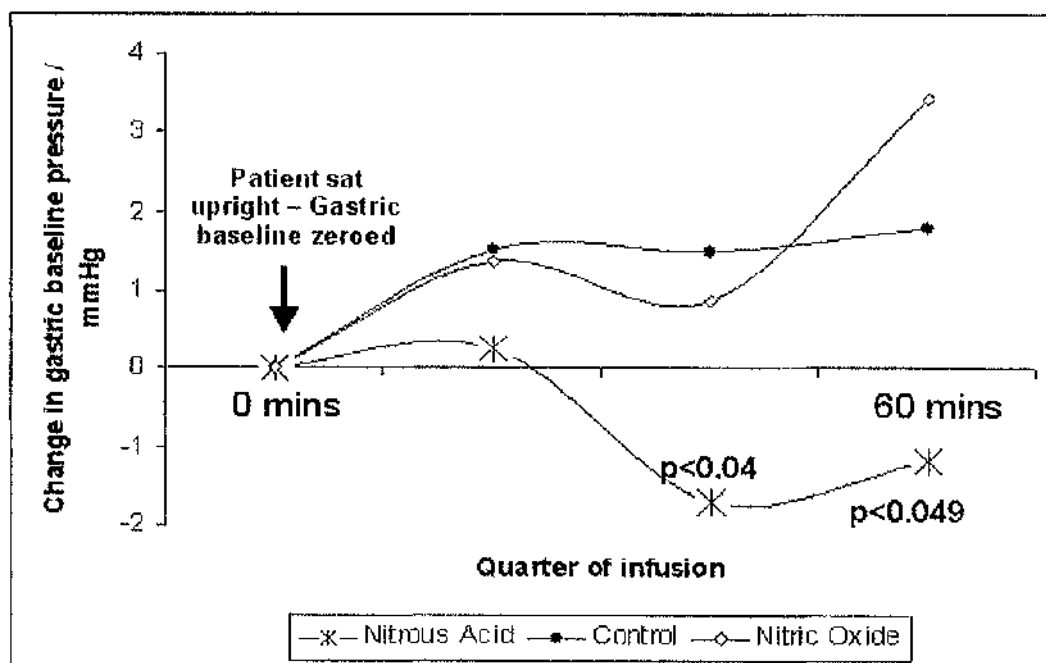


Figure 6.8: Intragastric pressure relative to immediate post-meal value during the third quarter of the infusion – ANOVA $p < 0.006$. Bars indicate mean values.

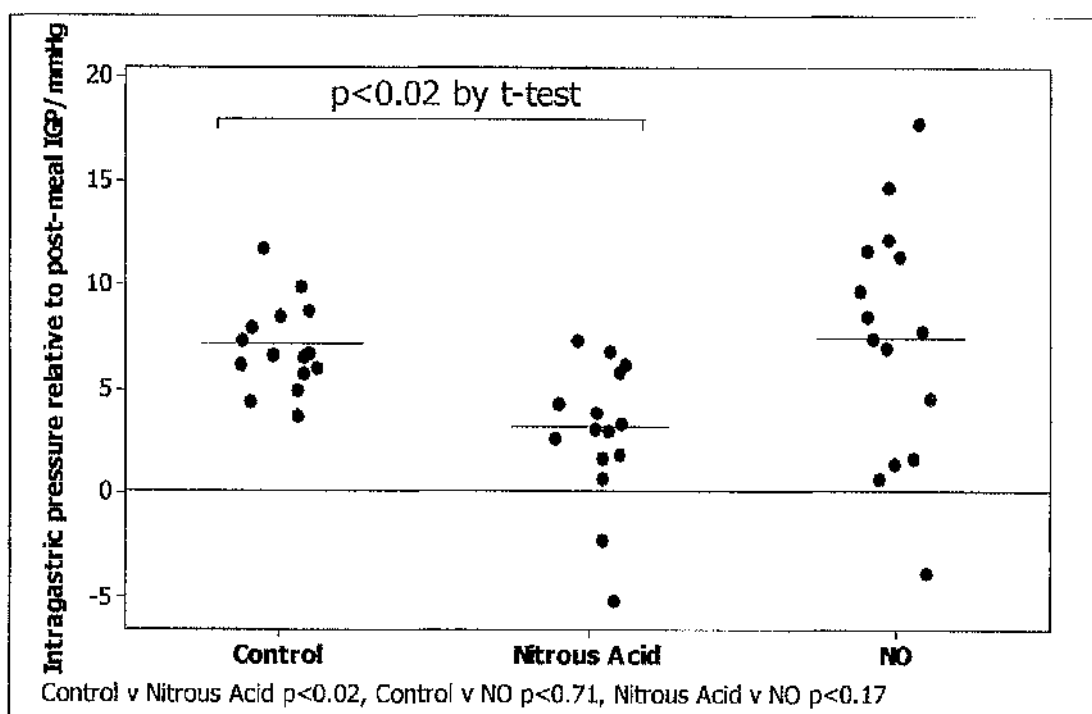


Figure 6.9: Comparison of IGP relative to immediate post-meal IGP during the fourth quarter of the infusion. Bars indicate mean values.

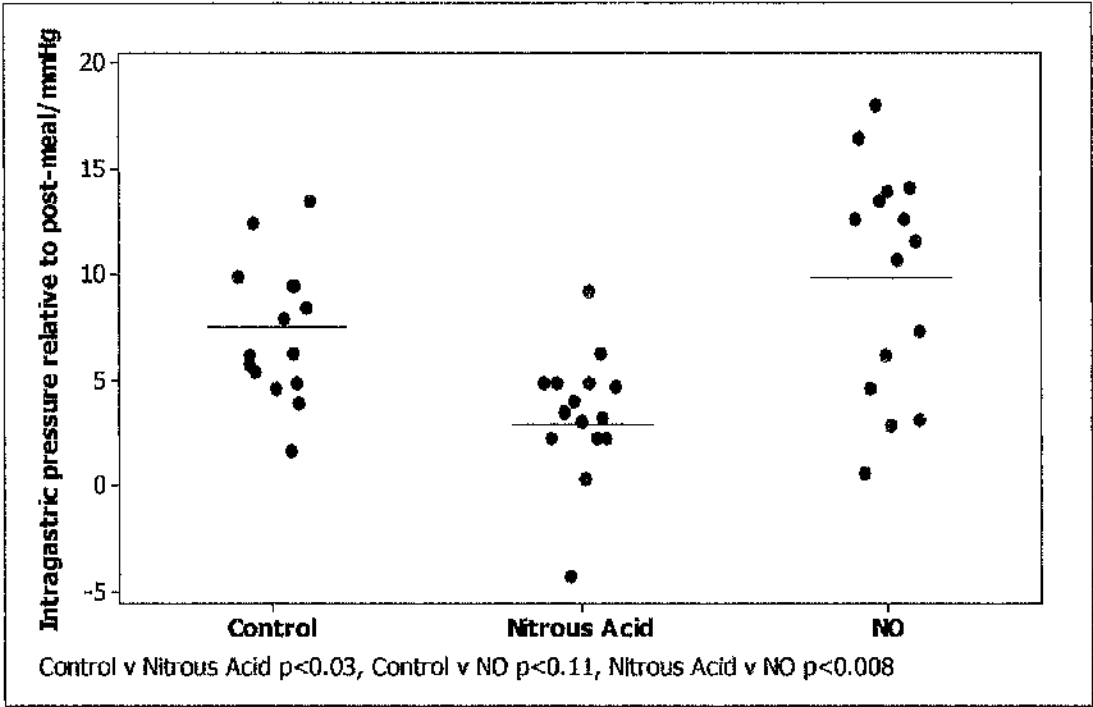


Figure 6.10: Comparison of IGP relative to fasting IGP during the third quarter of the infusion. Bars indicate mean values.

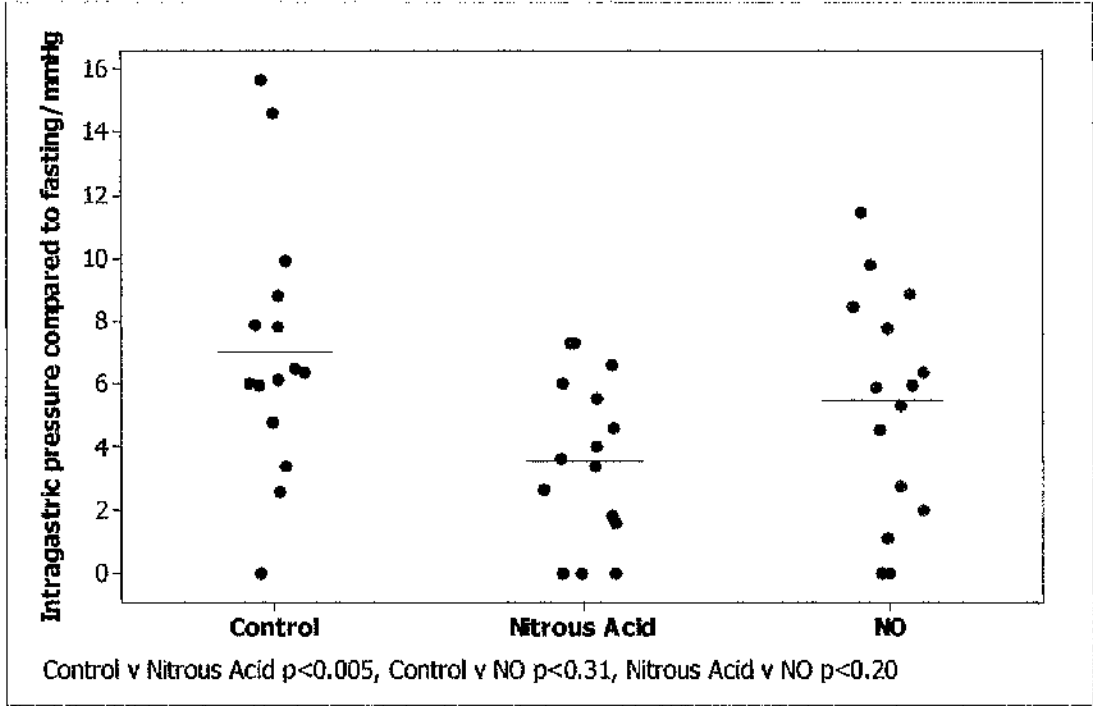


Figure 6.11: Comparison of IGP relative to fasting IGP during the fourth quarter of the infusion. Bars indicate mean values.

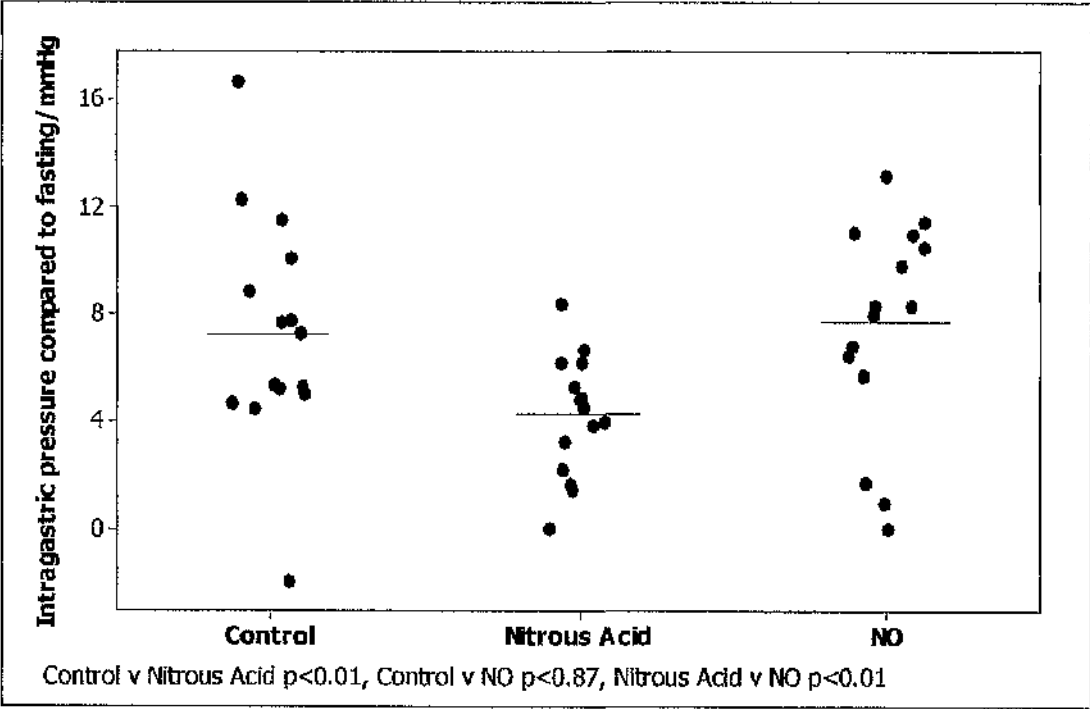


Figure 6.12: Length of the high pressure zone (HPZ) and abdominal sphincter length (AL) fasting and immediately following a meal. Bars indicate mean values.

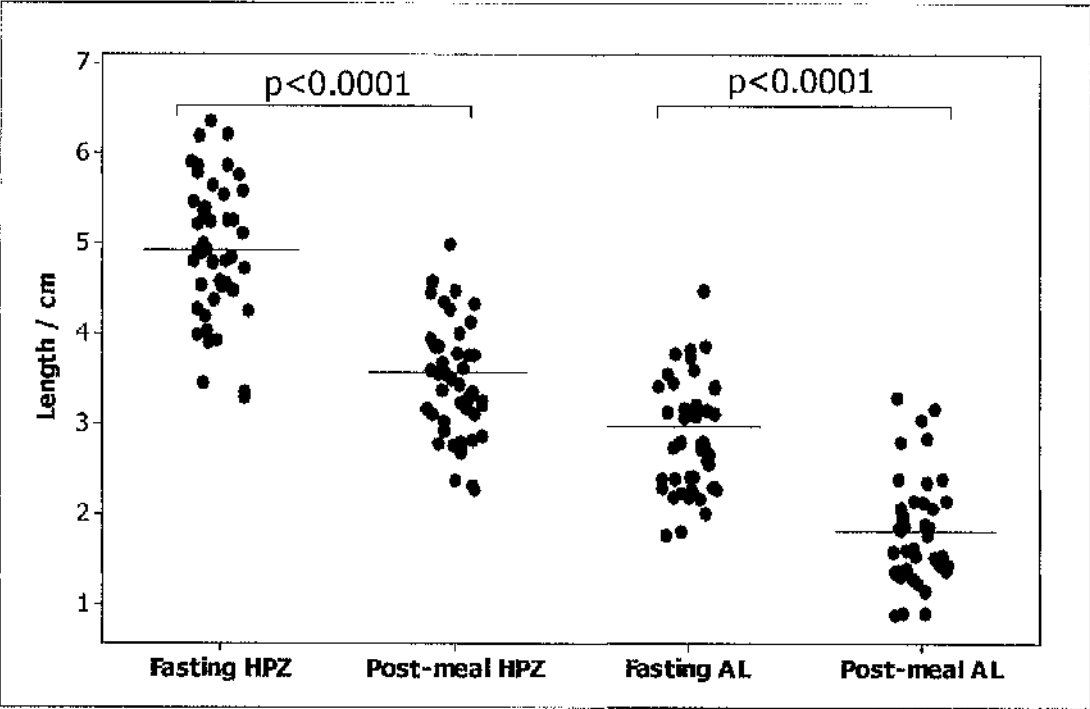


Figure 6.13: Maximum end expiratory pressure (MEEP) fasting and immediately following a meal. Bars indicate mean values.

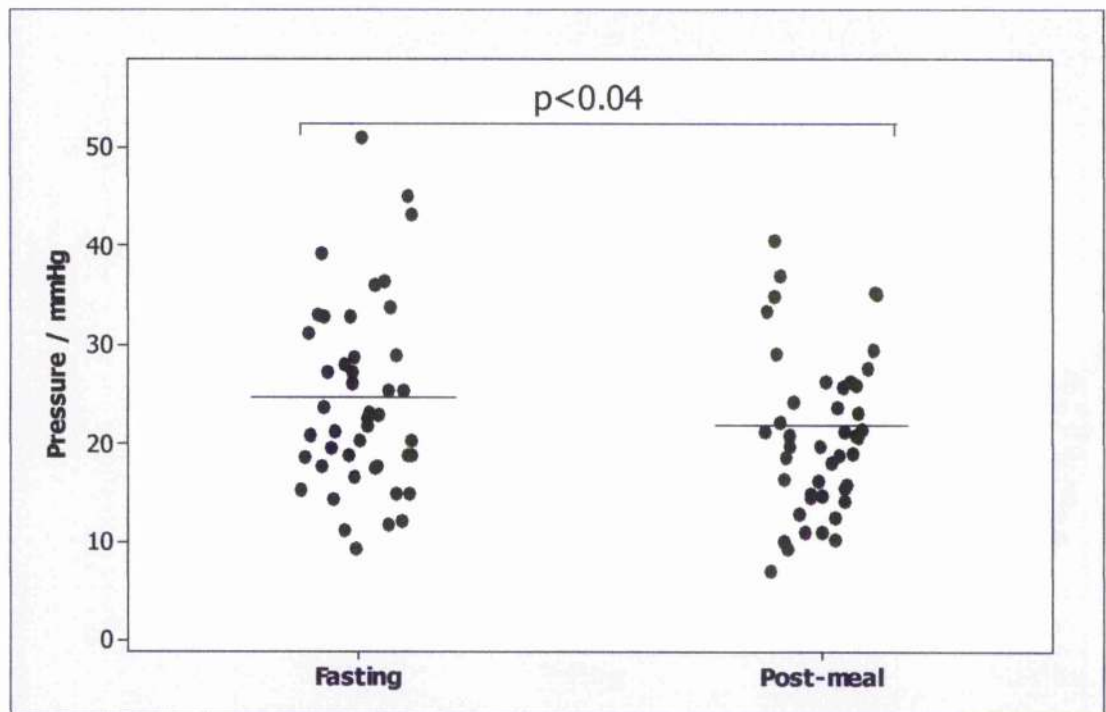


Figure 6.14: Mean positions of the upper and lower borders of the high pressure zone (HPZ) and the respiratory inversion point (RIP), (n=45).

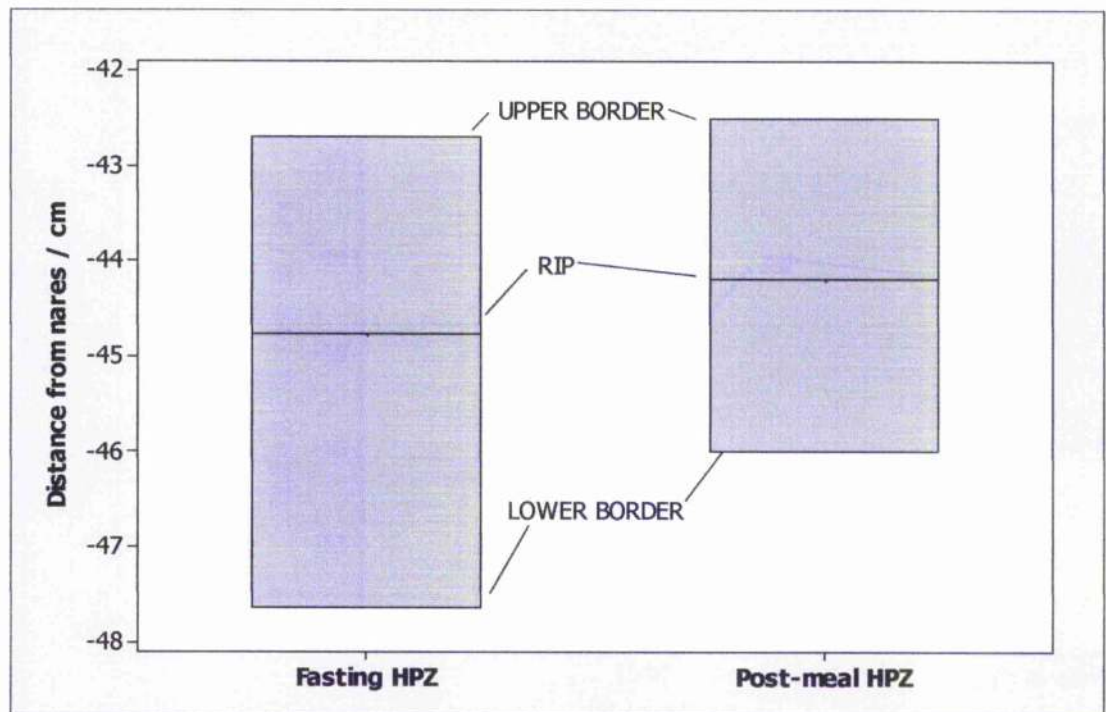


Figure 6.15: Comparison of each solution on LOS total length. Bars indicate mean values.

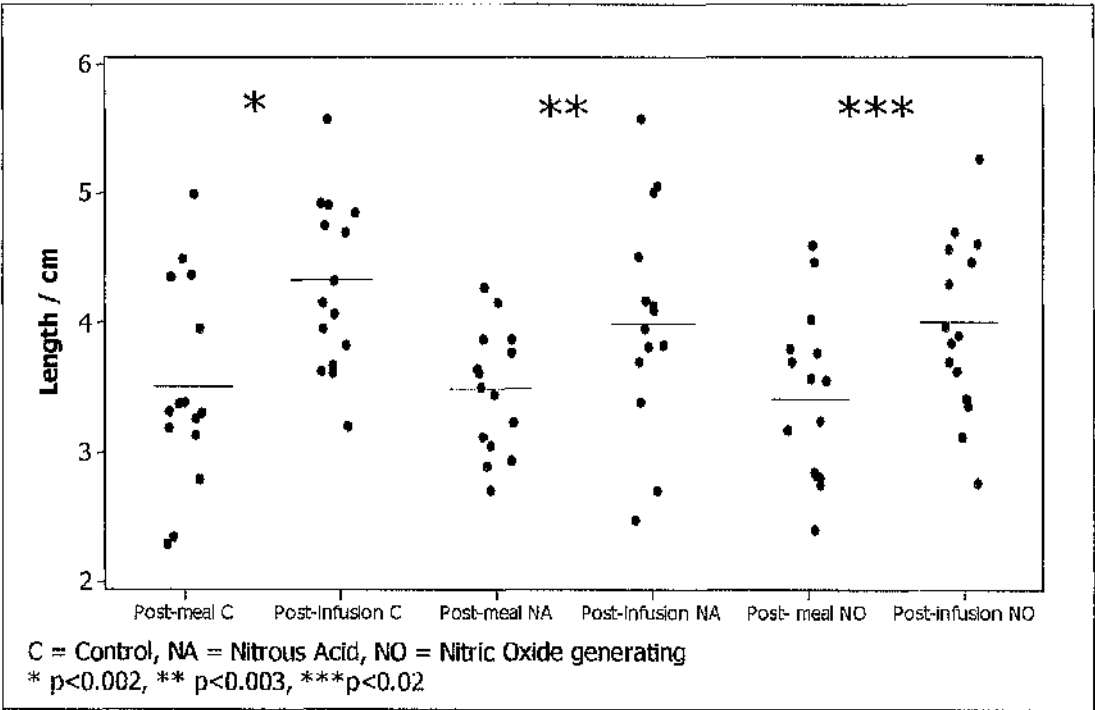


Figure 6.16: Comparison of each solution on LOS abdominal length. Bars indicate mean values.

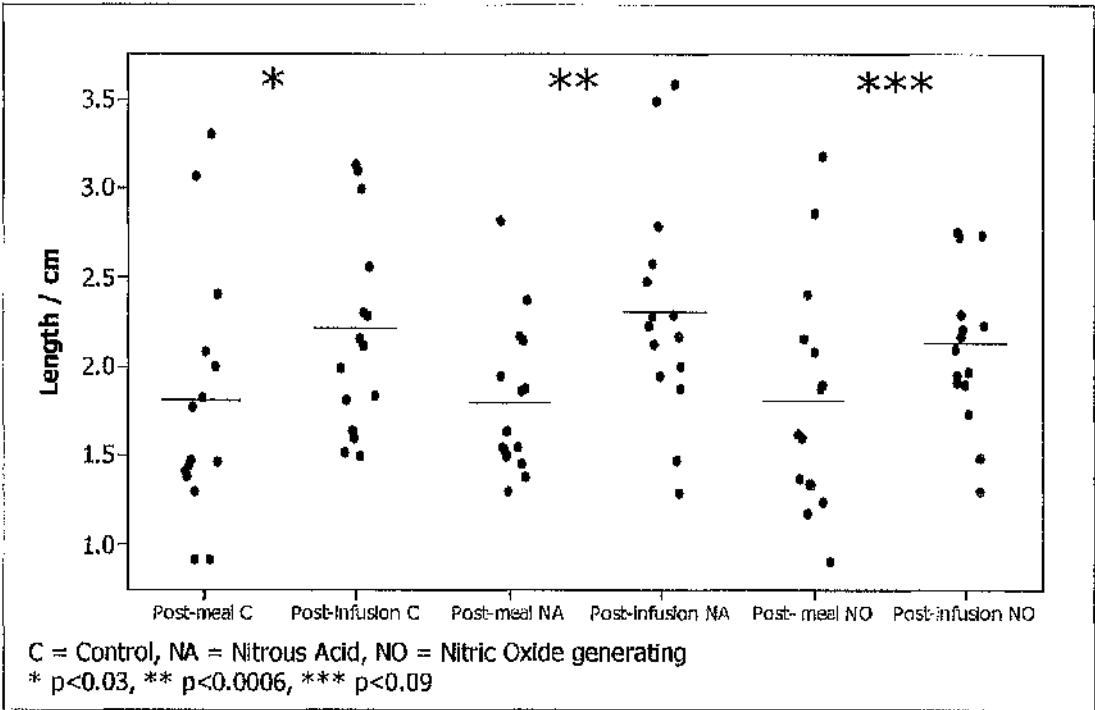


Figure 6.17: Location of the pH step-up point relative to the fasting high pressure zone (HPZ) and respiratory inversion point (RIP), (n=45).

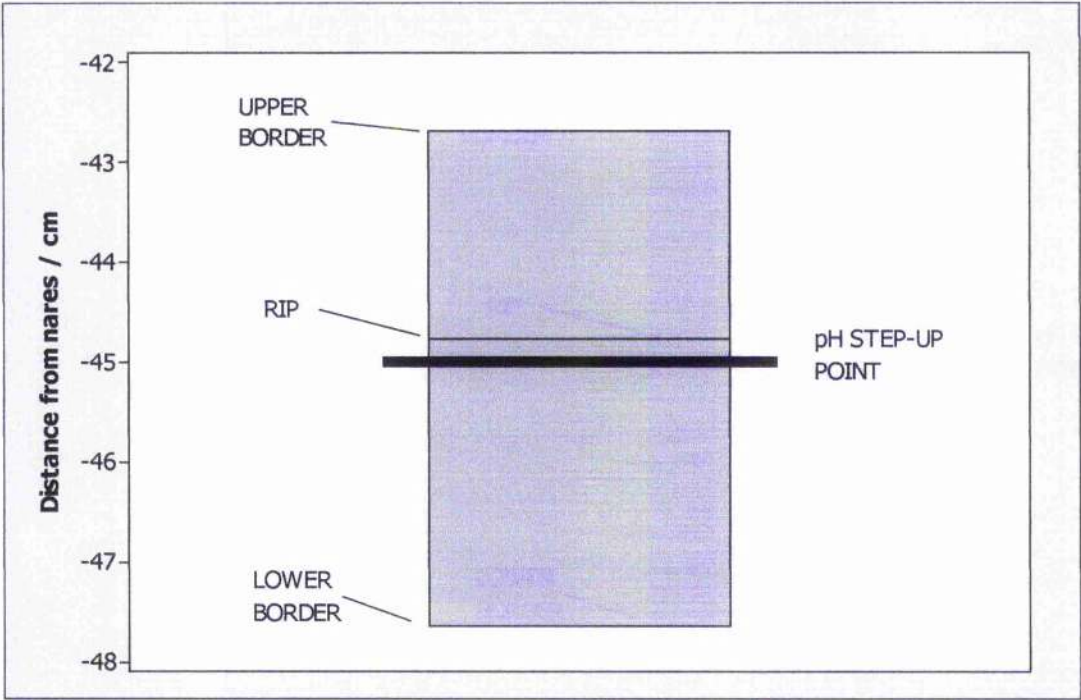


Figure 6.18: Position of distal pH probe relative to upper border of HPZ. Bars indicate mean values.

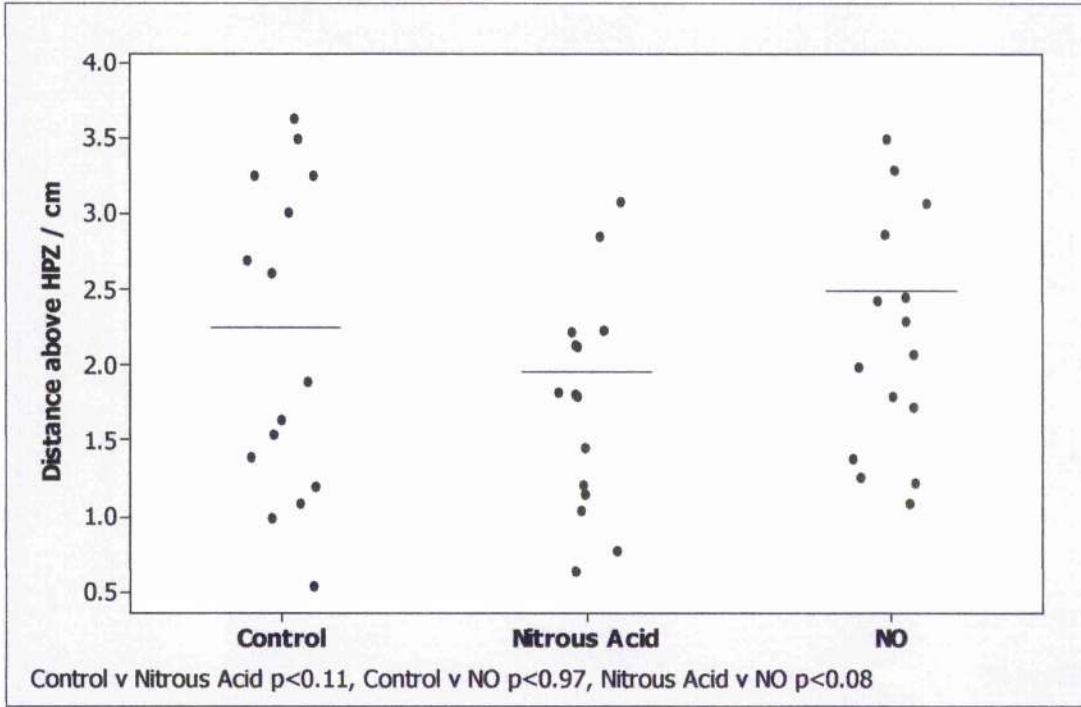


Figure 6.19: Proximal pH probe – % time pH<4. Bars indicate mean values.

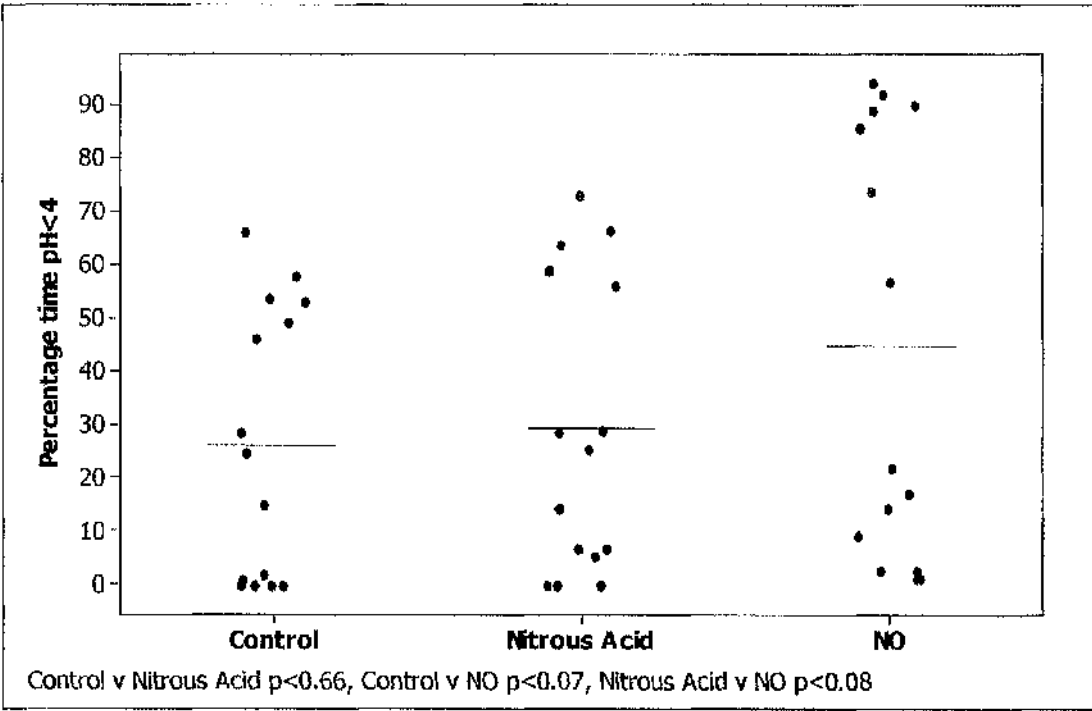


Figure 6.20: Proximal pH probe – total number of reflux episodes. Bars indicate mean values.

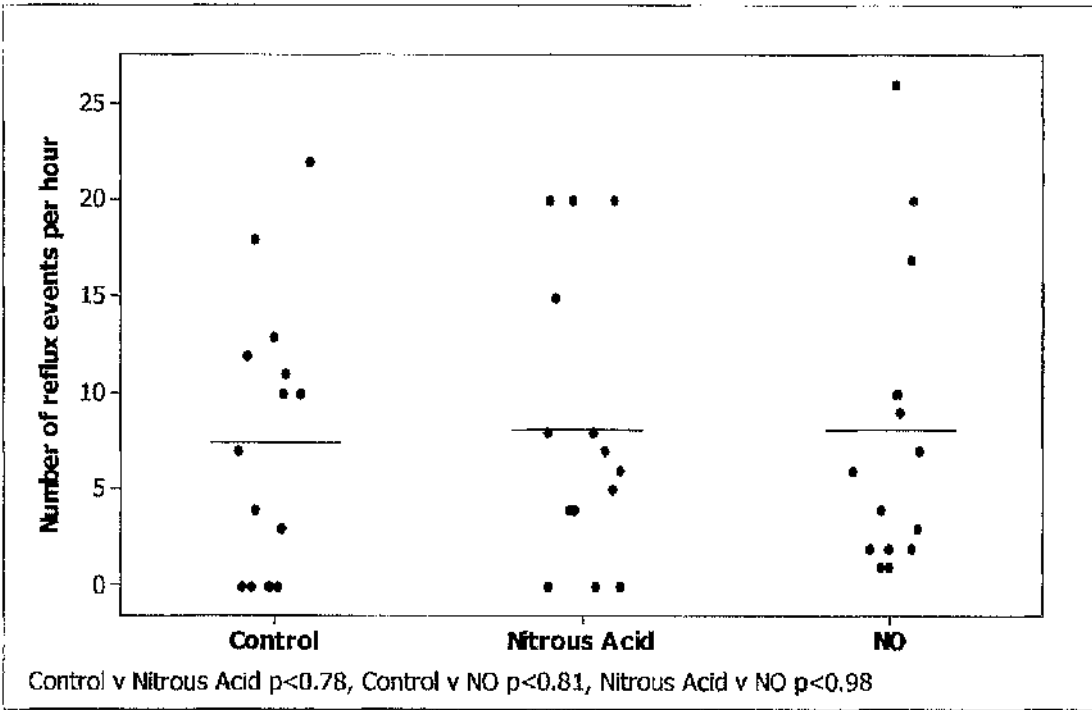


Figure 6.21: Proximal pH probe – mean duration per reflux event. Bars indicate mean values.

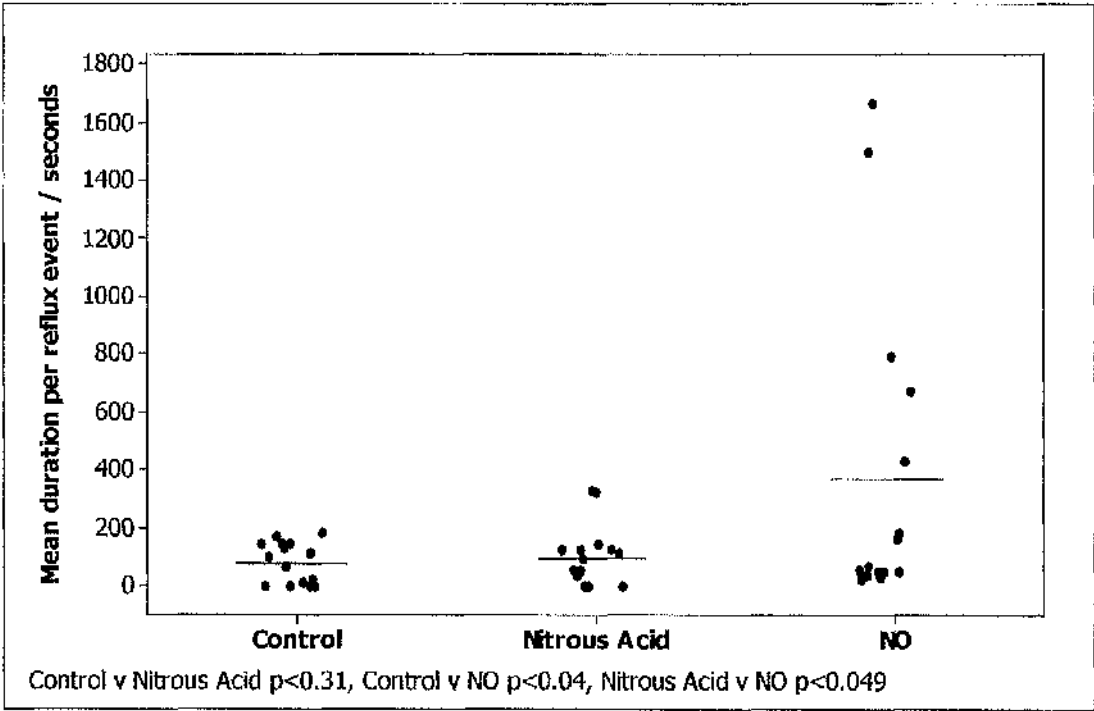


Figure 6.22: Distal pH probe - % time pH<4. Bars indicate mean values.

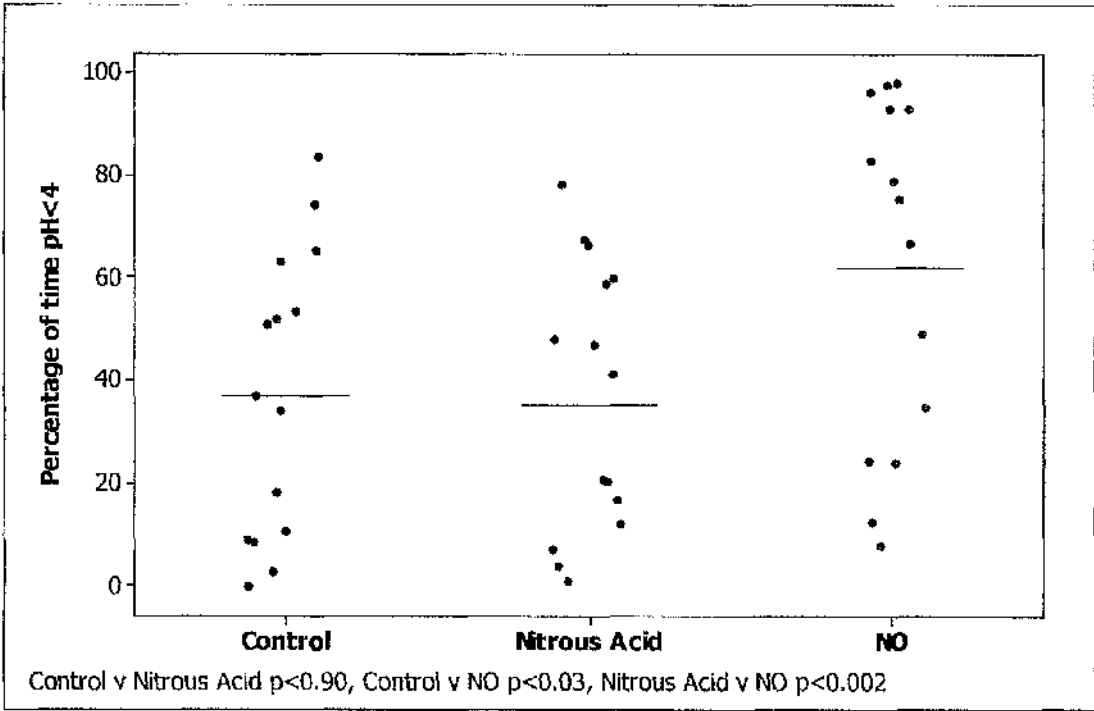


Figure 6.23: Distal pH probe – total number of reflux episodes. Bars indicate mean values.

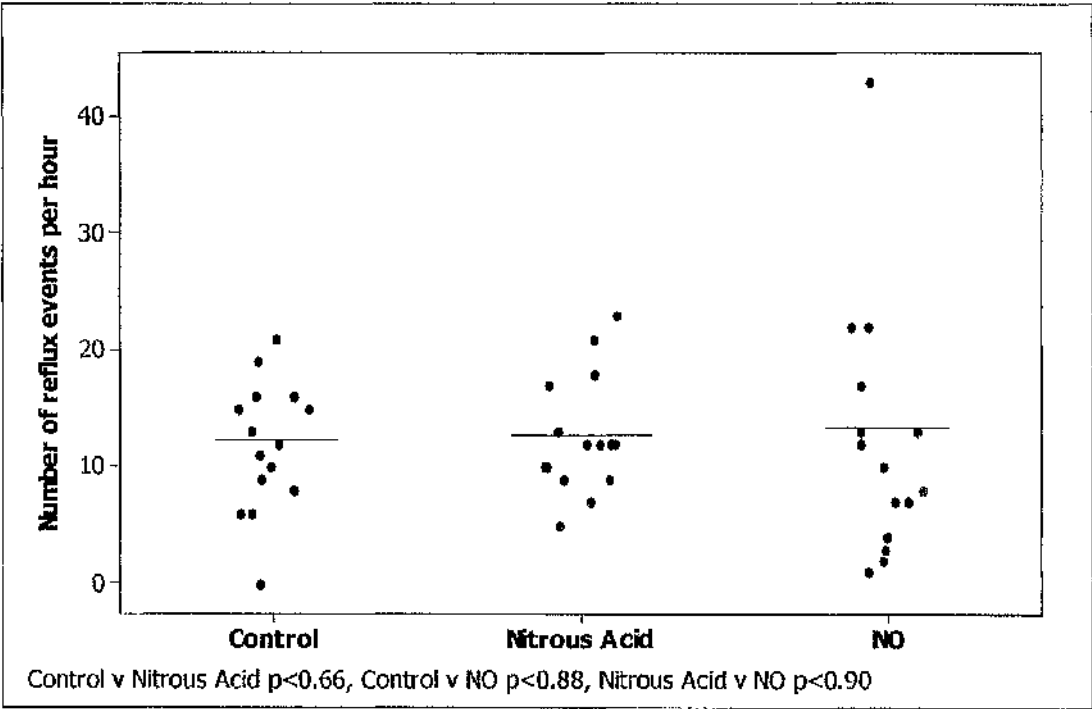
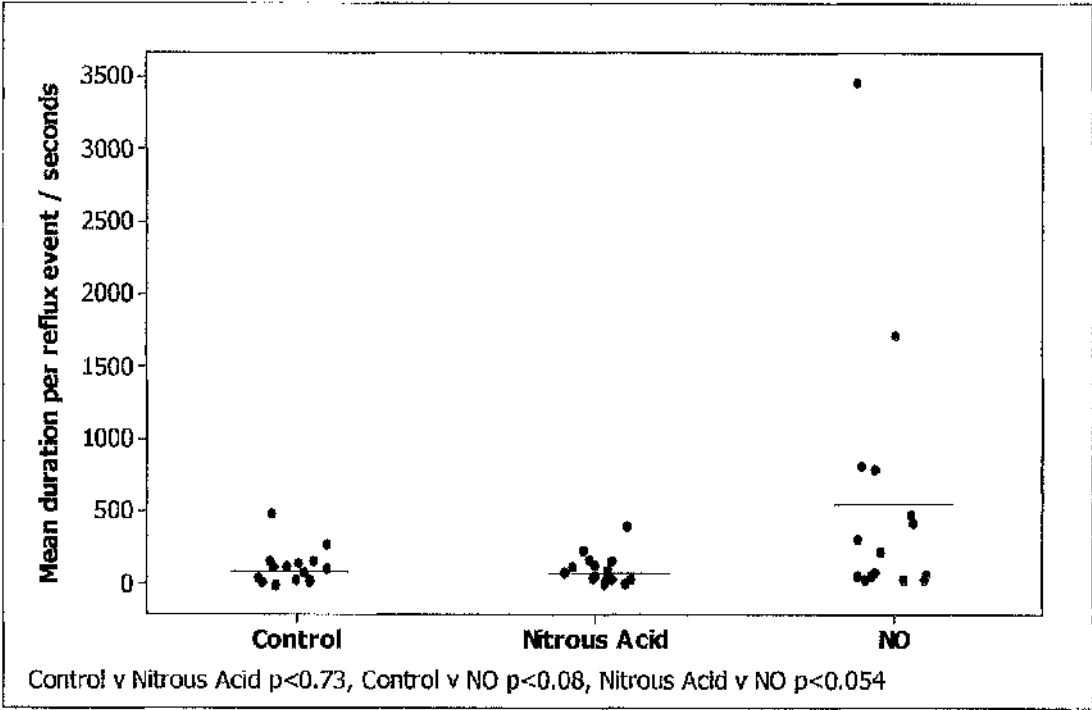


Figure 6.24: Distal pH probe – mean duration per reflux event. Bars indicate mean values.



Discussion

Chapter 7

7.1 Discussion:

This series of studies and experiments gives more insight into the potential physiological roles of dietary nitrate and salivary nitrite. GORD and the relatively overlooked phenomenon of short-segment reflux are clearly multi-factorial processes. This can lead to difficulty in deciphering the exact contribution of dietary nitrate and salivary nitrite to each condition. From this set of experiments it is plausible to assume that salivary nitrite could contribute to both long and short-segment reflux.

GORD is traditionally associated with long-segment reflux into the oesophagus. Refluxed luminal contents are poorly neutralised and clearance of luminal acid is reduced. However, lower LOS resting pressures are also contributory, but not a necessity. Short-segment reflux disease is one of the hypotheses for the generation of intestinal metaplasia at the gastric cardia, and perhaps even the distal squamous mucosa. Whether this is the precursor to short-segment Barrett's and perhaps even adenocarcinoma of the gastric cardia, is not clear.

These findings do tend to link together with the luminal chemistry. Clearly the most information from this work has been yielded from the 'meal' related studies (Chapter 6). Physiologically, reflux occurs even in healthy volunteers. However, the innate mechanisms, such as secretion of luminal bicarbonate, swallowing of saliva and clearance by secondary peristalsis, prevent both perception of reflux and damage from occurring. There is also, perhaps, a range of severity of physiological reflux. This may put healthy subjects at risk of transferring to a more GORD-like state if the luminal chemistry of the refluxate is able to dictate, in some capacity, whether

mucosal damage will occur. A healthy subject is unlikely to be aware of this as the majority of reflux events in healthy subjects are asymptomatic.

Our control arm of the reflux studies did show a variation in the degree of physiological reflux between our 15 subjects. All were asymptomatic and may even have had DeMeester scores considered to be pathological on formal 24-hour pH testing. This, however, was not performed. The silastic tube experiments appeared to back up the reflux data in the 2 subjects that we studied. This was by showing different oesophageal exposures to NO, the solution of which was delivered to the distal LOS, and which could be used as a surrogate marker of evidence of reflux.

The NO arm of the meal study demonstrated that high amounts of luminal NO appear to significantly impair oesophageal clearance, which as already discussed, is a major characteristic of GORD. This must be approached with a little caution, as must all of our results, due to us modelling the physiological mechanisms, rather than using them directly. That is to say, NO was delivered to the physiological site, with blockade of patient NO generation by avoidance of nitrate containing foods and eradication of buccal bacteria, rather than being intrinsically generated from swallowed salivary nitrite in the lumen. This was important to do for several reasons. Compliance of low nitrate ingestion can be problematical. Also, the conversion of nitrate to nitrite by the bacteria on the dorsum of the tongue is outwith our control.

In order to make everything level, subjects were dosed with the same amount of nitrite at their LOS. This was after all, a pilot study, attempting to prove a concept. Delivery of the nitrite in the test solution, was also non-physiological. Saliva is swallowed as boluses and delivered to the distal

oesophagus and LOS in this fashion, not as a continuous infusion. It is difficult to design flawless *in vivo* studies of this nature. It is important to have as few variables as possible, particularly if the physiological change that is being looked for is potentially very small indeed.

In addition, the viscosity of the carrier fluid, which was distilled water, is less than saliva, so the ability of the fluid to remain at the cardia region is reduced. Volume of fluid perfused is also questionable. Normal gastric secretion totals 1.5-2.0 litres per day. The majority of this occurs during meal times following gastrin release. We infused 400mls of test solution into the LOS region over the course of an hour. This again, is not entirely physiological. The reasons for needing to infuse such a large volume was down to the NO arm of the study. If the concentration of nitrite was any greater in the solution, which would have allowed volume reduction, then NO would have come out of solution in an obvious fashion. This would have filled the manometry system with NO gas bubbles, greatly affecting the performance of the pressure sensors and also effectively visually unblinding the study arm.

So, it was with a little reluctance that this volume was used, but it was at the time, the only plausible way that we could deliver such a high amount of NO. Delivering nitrous acid and ascorbic acid separately to the cardia would also have given unpredictable NO generation. The particular manometry catheter used (2282, Mediplus, UK) was also selected for several reasons. It gave the ability to deliver solutions to the lumen of the LOS due to the location of the distal ports. A sleeve system would have given accurate LOS measurements, but the solutions would have run-off distal to the LOS rather than within it. Also, the silicone rubber material from which a Dent

sleeve catheter is manufactured is very effective at letting NO pass through. Therefore, any NO generated in the syringe, even at high flow-rates, will pass out into the atmosphere before reaching the subject. The Mediplus catheter is made from medical grade PVC and retains the majority of NO within the system allowing adequate delivery within the subject.

Analysis of all healthy subject data in this thesis indicates that the important areas where statistically significant data have been obtained, occurred during the times of continuous monitoring, rather than single time points. These include the effects on oesophageal acid exposure, IGP and TLOSrs. In the earlier fasting study (Chapter 5), no effect was seen on resting MEEP following luminal NO. However, this does not definitely exclude an effect on the MEEP with NO. It may be that the protocol is not correctly designed to detect it. This may be relevant with the $T_{1/2}$ of NO being merely seconds. Also, subtle effects may be occurring at the LOS, in all of our studies, which are limited to the mucosal area. Our *in vivo* protocol or equipment may not be sensitive enough to detect these changes.

Carditis and intestinal metaplasia of the gastric cardia are very likely to be associated with bathing of the mucosa by acid, pepsin, proteolytic juice and bile. Intestinal metaplasia of the cardia is becoming more common in *H. pylori* negative subjects (253), in whom there is a definite increased risk of junctional adenocarcinoma. Whether a *H. pylori* positive state protects by altering the ascorbic acid availability, is not clear. Due to its location, reflux to this area is normally asymptomatic and as such, adenocarcinoma of the GOJ is not as strongly associated with a history of reflux as distal oesophageal adenocarcinomas (254).

7.2 Future work:

There are various avenues as to how this work could, and should, be taken forwards. It may be that on a population level, the prevention of disease (ie: primary prevention) in the future, will be of greater importance. Therefore, continuing work in healthy subjects, with attention to *H.pylori* status would be the way forward. In this asymptomatic group, disease at the SCJ is probably the most important. For patients with GORD, the best available medical therapy is acid suppression with proton pump inhibitors. There is also some expanding evidence for baclofen (129). Dietary modification is rarely followed and helps only approximately 20% of patients. However, there is some rationale for reducing meal size and increasing meal frequency. As the understanding of factors causing disease progression in GORD, and particularly those patients with Barret's oesophagus, are poorly understood, it is equally correct to focus these studies on this population.

There are several reasons, in fact, why this may be more appropriate. As patients with existing upper GI disease, they may be more sensitive to the effects of luminal nitrite chemistry. This may be related to either the differing properties of a columnar-lined oesophagus or the potentially more penetrating effects of the chemistry in the breached mucosa of oesophagitis. Penetration or diffusion of these luminal chemicals may even be important in the columnar-lined oesophagus, as the epithelium is only one layer thick, compared to the naturally occurring partially keratinised stratified squamous equivalent.

Permeation of NO or even refluxed nitroso-thiols into the lumen of the inflamed oesophagus, may cause relaxation of exposed or more susceptible muscularis mucosae. Physiological contraction of this thin muscular layer

normally acts as a protective mechanism, following a breach of the mucosa. A similar event would be expected post-endoscopic biopsy or even around ulcerated tissue.

One way to pursue this work is in hiatus hernia patients, with oesophagitis, and preferably those with Barrett's oesophagus. Hernia patients' results could prove very enlightening due to their anatomy. Due to the separation of the diaphragmatic crura from the LOS, this would give a better indication of effects seen on sphincter morphology and pressure. For the reasons previously discussed regarding day-to-day variability of LOS pressure, this type of study may prove unachievable. The pressures found in the LOS of hernia patients are lower than normal. Taking into account the variability of resting LOS pressures and desire to pick up small changes, it may well be that any effects may be too small to ever define.

It would be ideal if accurate data could be collected to assess oesophageal acid clearance by both pH monitoring and impedance after a meal, with concurrent oesophageal body, LOS and gastric body motility recording. Subjects would need to be off all acid suppression according to a strict protocol and follow both a low-nitrate diet and mouthwash regime as previously described. Also, since this work was carried out, high-resolution manometry (255;256) has evolved as a readily available resource. 'High resolution pH' has also been prototyped in our unit (unpublished work at time of writing). These modalities would remove some of the complications presented by our study design as well as providing, previously unavailable information. The problem with over-complicating protocols is that excessive luminal instrumentation can interfere with normal physiological function, as can the introduction of large volumes of luminal chemicals. A simpler

experiment would therefore be to examine the effect of swallowed nitrite on reflux and intra-oesophageal pH after a meal.

In order to assess the sensitivity of the NA mechanism, a dose-response effect, could be looked at in a randomised, double-blinded fashion. Only then will we have a better grasp of whether more day-to-day physiological levels of salivary nitrite can be implicated in the potentiation of upper GI disease. One problem with our healthy subject studies, and a reason for stressing their upper GI tract with so much NA and NO, was that it was unlikely that a big effect would be seen in this population. It was, however, interesting to see statistically significant data from a study size of $n=15$. Regardless, the maximum dose used, equivalent to 24 hours post-prandial nitrate exposure over a one-hour period, was entirely appropriate.

The role of antioxidants in the diet has been discussed already (section 6.12). The most commonly available antioxidant available in the upper GI tract is ascorbic acid. This is due to both its secretion into the gastric juice and also because of its frequent ingestion in meals. The fasting profile of ascorbic acid in the stomach has been analysed (241). In the fasting state it showed that there is a gradient of $13.0\mu\text{moles/litre}$ (SEM $6.1\mu\text{moles/litre}$) at the cardia to $86\mu\text{moles/litre}$ (SEM $29\mu\text{moles/litre}$) at the antrum. This may impact on luminal NO production. Boluses of nitrite are more likely to give higher levels of NO, with replenishment of ascorbic acid following its reduction. The time for proximal gastric ascorbic acid levels to replenish are not known. Post-prandial intragastric ascorbic acid levels, either related to gastric secretion or meal ingestion are also not known.

Upper GI disease states such as Barrett's oesophagus and adenocarcinoma can take decades to develop, perhaps in a small, step-wise fashion. This, theoretically, could be related to recurrent effects of luminal nitrite chemistry in the post-prandial setting. However, further negative results do not completely exclude nitrite as a co-factor in upper GI disease. If further effects were seen in hernia patients then removal of the acid, probably with proton pump inhibitors, to show the importance of acidifying the nitrite, could be taken further. The acid-pocket has been demonstrated as an unbuffered region of gastric acid-following a meal. The pH of this area appears to mirror that of the oesophageal refluxate. Similarly, for hiatus hernia patients, some work carried out in our Unit has shown that hiatus hernia patients have a larger unbuffered region of post-prandial acid, related to the hiatal sac (257). Therefore, targeting therapies at modifying acid secretion of the hiatal sac and proximal stomach may prove more beneficial in the long term.

Further work will be funded with a substantial grant awarded by the Scottish Office, December 2006.

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